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Casein kinase II interacts with the bZIP domains of several transcription factors

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ABSTRACT

Casein kinase II (CKII) is thought to regulate a broad range of transcription factors, but its mode of action is not well characterized. We previously showed that CKII is co-purified with the ATF family of transcription factors using DNA-affinity latex beads. Here we report a functional and physical interaction between CKII and transcription factors. We demonstrate that CKII binds through its catalytic α and α′ subunits to the basic leucine zipper (bZIP) DNA-binding domains of many transcription factors, including ATF1. Kinetic analysis using a surface plasmon resonance sensor suggests that CKII loosely associates with ATF1 *in vivo*. Deletion of the bZIP domain of ATF1 markedly reduces its phosphorylation by CKII, suggesting that the bZIP recruits CKII to the vicinity of the target site. ATF1-CKII complex is also formed on DNA. Using CKIIα fused to a heterologous DNA-binding domain, we also demonstrate that CKII, when bound to DNA, efficiently phosphorylates its substrate, which is bound to the same DNA molecule. Taken together, CKII may regulate transcription (and possibly other events) by phosphorylating proteins on DNA.

INTRODUCTION

Protein phosphorylation is a global mechanism for transcription regulation. Phosphorylation can affect transcription factors by changing their DNA-binding activity, subcellular localization or ability to interact with other components of the transcription apparatus (1). In addition, phosphorylation of RNA polymerase II (pol II) within the C-terminal domain (CTD) of its largest subunit may trigger transition of the transcription reaction from the initiation to the elongation phase (2).

Among many protein kinases that phosphorylate the transcription apparatus, casein kinase II (CKII) is of particular interest. CKII is a ubiquitous, heterotetrameric serine/threonine protein kinase that is composed of two catalytic (α or α′) and two regulatory (β) subunits. CKII phosphorylates a variety of transcription factors *in vivo* and *in vitro* (3). Transcriptional activators [such as c-Myc (4), c-Jun (5), SRF (6), Sp1 (7), VDR (8) and c-Myb (9)], repressors [I-κB (10) and Cut (11)], basal factors [UBF (12), TFIIIB (13)], cofactors [NC2/Dr1 (14) and PC4 (15)] and viral transactivators [adenovirus E1A (16), HPV E7 (16) and HSV VP16 (17)] are phosphorylated and their activities are either positively or negatively modulated by CKII. However, since CKII is a constitutively active enzyme, it remains elusive as to how phosphorylation might have any regulatory role, for instance, in a temporal or a signal-responsive manner.

Previously, we purified several ATF/CREB transcription factors using DNA-affinity latex beads bearing ATF sites of the adenovirus E4 promoter (18). Using the latex beads, we co-purified a protein kinase activity that can efficiently phosphorylate ATF1 (19). The kinase did not bind DNA or the latex beads by itself, but it bound ATF1 *in vitro* (19). Biochemical and immunological data indicated that this ATF-associated kinase is identical to CKII (19). To reveal the molecular basis for the co-purification, here we have analyzed the interaction between CKII and transcription factors. We demonstrate that the catalytic α and α′ subunits of CKII directly interact with the basic leucine zipper (bZIP) DNA-binding domains of ATF1 and many other transcription factors. We also demonstrate the functional significance of their interaction. Finally, a possible model for the mechanism of transcription regulation by CKII is presented.

MATERIALS AND METHODS

Plasmids

A series of plasmids expressing ATF1 derivatives were constructed as follows. To generate the C-terminal deletion mutants of ATF1, pGEM-ATF1 (44) was digested with *Sal*I and *Bam*HI, *Sca*I or *Xho*I and cloned into pGEX-5X-3 (Pharmacia), to give mutA, mutB and mutC, respectively. For mutE, pGEX-ATF1 (19) was digested with *Bam*HI and *Pst*I and the small fragment was ligated with the *Bam*HI–*Pst*I fragment of pGEX-5X3. For mutG, pGEM-ATF1 was digested with *Bam*HI and *Sca*I and re-circularized. The resulting plasmid was then digested with *Sal*I and *Eco*RI and re-cloned into pGEX-2T (Pharmacia). For mutF, pGEM-ATF1 was digested with *Sca*I and *Eco*RI and cloned into pGEX-5X-3. For mutJ, pGEX-ATF1 was digested with *Bam*HI and *Xho*I and re-circularized. To construct other ATF1 mutants, the DNA fragments encoding amino acids 1–214 (ΔbZIP), 214–271 [bZIP(ATF1)], 214–239 (BR) and 240–271 (LZ) of ATF1 were amplified by PCR using pairs of appropriate primers with the *Bam*HI and *Eco*RI sites, respectively. They were digested with *Bam*HI and *Eco*RI and cloned into the *Bam*HI and *Eco*RI sites of pGEX-5X-3. For bZIP(L248P), Leu248 within the bZIP domain

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was mutated to proline by the PCR overlap extension method (45). Two mutagenic primers, 5′-GTTGAGTCCCGGAAAATC-AAAAT-3′ and 5′-ATTTGATTTTCCGGGACTGCAAC-3′, were used (mutated nucleotides underlined).

To generate plasmids expressing bZIP(CREB), bZIP(CRE-BP1), bZIP(Fos) and bZIP(Jun) fused to glutathione S-transferase (GST), DNA fragments encoding amino acids 268–327 of human CREB, 351–415 of human CRE-BP1, 139–211 of rat c-Fos and 222–331 of human c-Jun were amplified by PCR and cloned into pGEX-5X-3.

The complete cDNA clones of human CKIIα, α′ and β were provided by Dr E. G. Krebs (46). To construct pGEX-CKIIα and pGEX-CKIIβ, expressing GST-CKIIα and GST-CKIIβ, the complete cDNA fragments of CKIIα and β were amplified by PCR and cloned into pGEX-5X-3. To construct pET-CKIIα and pET-CKIIβ, expressing His-CKIIα and His-CKIIβ, pGEX-CKIIα and pGEX-CKIIβ were digested with *Bam*HI and either *Eco*RI or *Xho*I and cloned into pET-14b. For His-CKIIα′, the complete CKIIα′ cDNA amplified by PCR was cloned into pET-14b.

To construct an expression vector for Gal4-CKIIα, the PCR-generated fragment of Gal4 (1–147) and the full-length cDNA fragment of CKIIα were triple ligated with pET-14b. To construct an expression vector for LexA-S, a PCR-generated fragment encoding amino acids 1–87 of the LexA DNA-binding domain and a double-stranded oligonucleotide encoding amino acids 1963–1970 of the largest subunit of human RNA pol II were triple ligated with pET-14b.

Protein preparation

GST fusion proteins were expressed and purified from *Escherichia coli* using glutathione–Sepharose according to the manufacturer's instructions (Pharmacia). GST-ATF1 derivatives used for phosphorylation assay were further purified from gel slices by a denature–renature protocol (47). Histidine-tagged proteins were expressed and purified as described (48). ^{35}S-Labeled CKIIα and its mutants were synthesized *in vitro* using the rabbit reticulocyte lysate system (Promega) as described (19). Human CKII was purified from HeLa cells as described (19).

Transfection and immunoprecipitation assay

HeLa cells were maintained in MEM (Nissui) supplemented with glutamate and 10% fetal bovine serum. Expression plasmid pFLAG-ATF1 (10 µg) was transfected into 5×10^6 HeLa cells by the standard calcium phosphate method. After 48 h, the cells were lysed with lysis buffer (50 mM Tris, pH 7.9, 500 mM NaCl, 1% NP-40). The total cell lysate (800 µl) was incubated with 20 µl anti-FLAG M2 affinity gel (IBI-Kodak) for 2 h at 4°C. The immunoprecipitate was washed three times with 0.5× lysis buffer and eluted four times with 20 µl 0.5× lysis buffer containing 180 µM FLAG peptide (IBI-Kodak). The eluates were analyzed by immunoblotting using α-FLAG M2 monoclonal antibody and α-CKIIα monoclonal antibody (Boehringer Mannheim).

GST pull-down assay and DNA co-precipitation assay

About 5 µg GST fusion proteins were coupled to 20 µl glutathione–Sepharose by incubating for 30 min at 4°C. The beads were washed three times with NETN (20 mM Tris, pH 7.9, 100 mM NaCl, 1 mM EDTA, 0.5 % NP-40) and once with buffer A (50 mM Tris, pH 7.9, 20% glycerol, 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.1% NP-40). One to five microliters of rabbit reticulocyte lysate containing ^{35}S-labeled CKIIα or 100–200 ng bacterially expressed His-CKIIα, α′ or β was then incubated with the beads in buffer A for 1 h at 4°C. After washing five times with buffer A, the bound proteins were eluted with SDS sample loading buffer and resolved by 10% SDS–PAGE. ^{35}S-Labeled CKIIα was detected by fluorography. Bacterially expressed His-CKIIα, α′ and β were visualized by western blotting using anti-CKII rabbit serum (49).

A ^{32}P-labeled DNA fragment bearing an ATF site was prepared as described (50). The fragment was incubated either in the presence or absence of 100 ng His-ATF1 for 30 min at 30°C and binding of the mixture to GST derivatives was examined as above. The DNA fragment retained on the column was recovered by phenol–chloroform extraction and ethanol precipitation, separated by 6% native PAGE and detected by autoradiography.

Far-western blotting

Partially purified GST fusion proteins were resolved by 10% SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. The blotted proteins were denatured in buffer A containing 6 M guanidine–HCl (Gu-HCl) for 15 min and renatured by sequentially washing with buffer A + 3 M Gu-HCl for 10 min, buffer A + 1.5 M Gu-HCl for 10 min, buffer A + 0.75 M Gu-HCl for 10 min, buffer A + 0.375 M Gu-HCl for 10 min, buffer A for 30 min twice and buffer A containing 5% BSA for 1 h. All washings were performed at 4°C. The membrane was then incubated with 5 ml buffer A containing 25 µl rabbit reticulocyte lysate containing ^{35}S-labeled CKIIα for 3 h at 4°C, washed three times with buffer A and dried for fluorography.

Kinetic analysis using a surface plasmon resonance (SPR) sensor

Protein–protein interactions were measured using an SPR sensor system (BIAcore 2000). GST derivatives were fixed onto the sensor surface via anti-GST antibody. For immobilization of goat anti-GST antibody (BIAcore) to the sensor surface, standard amine coupling chemistry was used (51). Briefly, carboxymethylated dextran on the sensor surface of Sensor Chip CM5 (BIAcore) was activated with EDC and NHS. Goat anti-GST antibody (30 µg/ml, 50 µl) in coupling buffer (10 mM sodium citrate, pH 5.0) was applied to the activated carboxymethylated dextran surface. After immobilization, the remaining esters were deactivated by ethanolamine. The sensor surface was washed with 5 µl glycine–HCl (pH 2.2), to remove antibody which was not immobilized covalently. Subsequently, the *E.coli* lysates containing GST fusion proteins in running buffer (10 mM HEPES, pH 7.6, 120 mM KCl, 1 mM EDTA, 0.01% Tween 20) were applied to the immobilized anti-GST antibody. The GST fusion proteins were fixed to the sensor chip through antigen–antibody interactions. The reactions were adjusted so that approximately equimolar amounts of the GST fusion proteins were fixed. The remaining free antibody was masked with GST protein. The sensor surface was washed with 5 µl 2.0HKET (10 mM HEPES, pH 7.6, 2.0 M KCl, 1 mM EDTA, 0.01% Tween 20) to remove non-specifically bound proteins.

Running buffer was continuously passed over the sensor surface at a flow rate of 30 µl/min. The analytes, purified His-CKIIα diluted with running buffer, were injected and passed over the sensor surface. After injection, running buffer replaced the analytes. The association and the following dissociation

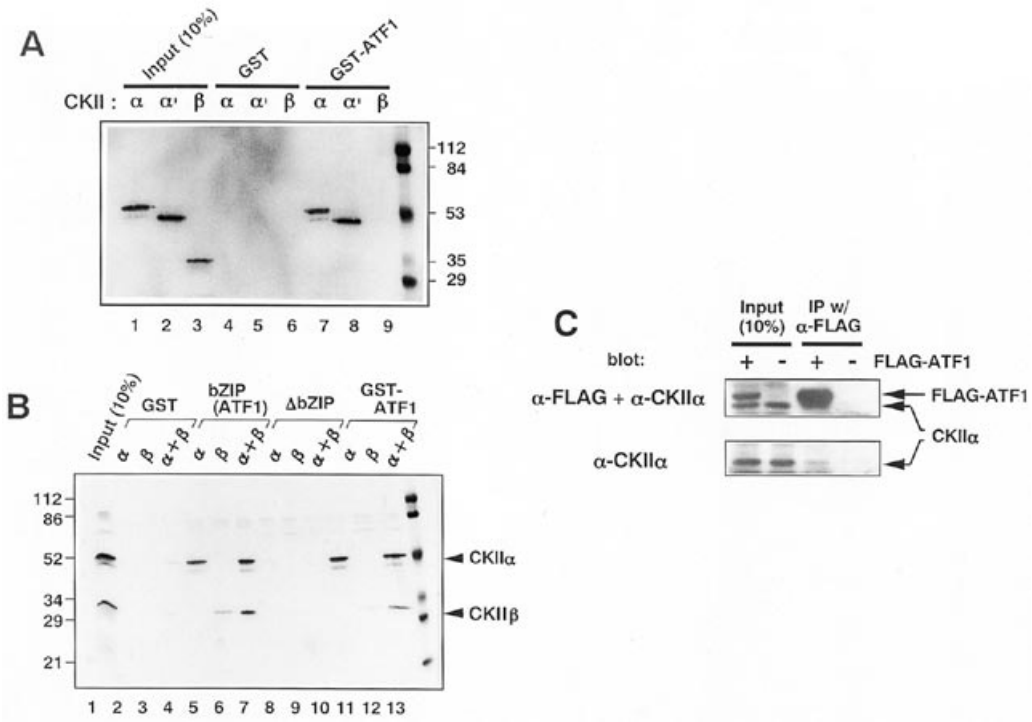

Figure 1. CKII directly interacts with ATF1 *in vitro* and *in vivo*. (**A**) Interactions between subunits of CKII and ATF1. All the subunits (α, α′ and β) of CKII were expressed as histidine-tagged proteins in *E.coli* and 100–200 ng protein were incubated with glutathione–Sepharose beads bound by 5 µg GST (lanes 4–6) or GST-ATF1 (lanes 7–9) for 1 h at 4°C. After extensive washing, the bound proteins were resolved by SDS–PAGE and detected by western blotting using anti-CKII rabbit serum. Ten percent of the input is shown in lanes 1–3. (**B**) Holo-CKII binds the bZIP domain of ATF1 *in vitro*. His-CKIIα alone (lanes 2, 5, 8 and 11), His-CKIIβ alone (lanes 3, 6, 9 and 12) or both (lanes 4, 7, 10 and 13) were incubated for 30 min at 4°C. Their interactions with the indicated GST fusion proteins were examined as described in (A). Lane 1 shows 10% of the input. (**C**) CKII-ATF1 interaction *in vivo*. Expression plasmid pFLAG-ATF1 (10 µg) was transfected into 5×10^6 HeLa cells by the standard calcium phosphate method. The cells were lysed and immunoprecipitated with anti-FLAG affinity gel. After extensive washing, the bound materials were eluted with FLAG peptide and the presence of FLAG-ATF1 and endogenous CKII were examined by western blot analysis.

reactions were detected in real time using the optical phenomenon of SPR (52). The SPR response is expressed as resonance units (RU). The transition of the RU value corresponds to the amount of analyte bound to ligand on the surface. At the end of each detection, the sensor surface was regenerated by injecting 10 µl 2.0HKET.

For the analysis, each sensorgram was subtracted from the response of the surface on which only GST was immobilized. The rate constants of the interactions were calculated by a non-linear analysis of the association and dissociation curves using the SPR kinetic evaluation software package BIAevaluation 2.1 (BIAcore). The kinetic data were interpreted in the context of the simple binding model: A + B = AB (53,54). The equilibrium dissociation constant was calculated from the values of association rate constant and single averaged dissociation rate constant, according the thermodynamic relationship: $K_d = k_d/k_a$.

DNA-dependent kinase assay

Oligonucleotides with a Gal4 binding site (5′-GATCCGGAG-TACTGTCCTCCG-3′) (55) and a LexA binding site (5′-TCGA-TACTGTATGAGCATACAGTA-3′) (56) and their complementary strands were synthesized. They were annealed, phosphorylated and inserted into the *Bam*HI and *Xho*I sites of pUC119 respectively. A plasmid carrying both binding sites was also constructed. They were digested with *Eco*RI and *Hin*dIII and the resulting fragments of ~100–200 bp were purified by native PAGE.

The DNA-dependent kinase assay was performed in 10 µl kinase buffer as follows. Gal4-CKIIα (1 nM), LexA-S (1 nM) and the indicated concentrations of DNA fragment containing Gal4, LexA or both sites were preincubated for 30 min at 30°C. When indicated, ethidium bromide was included in the mixture. Reactions were started by adding 10 µM ATP and 1 µCi [γ-^{32}P]ATP, incubated for 2 min at 30°C and stopped by adding SDS sample loading buffer. Phosphorylated LexA-S was separated by 12.5% SDS–PAGE and detected by autoradiography.

RESULTS

α and α′ subunits of CKII directly interact with ATF1

CKII is composed of two catalytic (α and α′) and two regulatory (β) subunits. To determine whether CKII directly interacts with members of the ATF family, we performed GST pull-down assays. ATF1 fused to GST (GST-ATF1) and all the subunits (α, α′ and β) of CKII with a histidine tag were expressed and purified from *E.coli*. As shown in Figure 1A, His-CKIIα and His-CKIIα′ equally bound GST-ATF1, but not control GST. In contrast, very weak, if any, binding of His-CKIIβ to GST-ATF1 was detected (Fig. 1A and B). Therefore, we conclude that the catalytic α and α′ subunits of CKII directly interact with ATF1. Since the CKIIα and CKIIα′ polypeptides are highly homologous (the first 330 amino acids are 88% identical), only CKIIα was examined further.

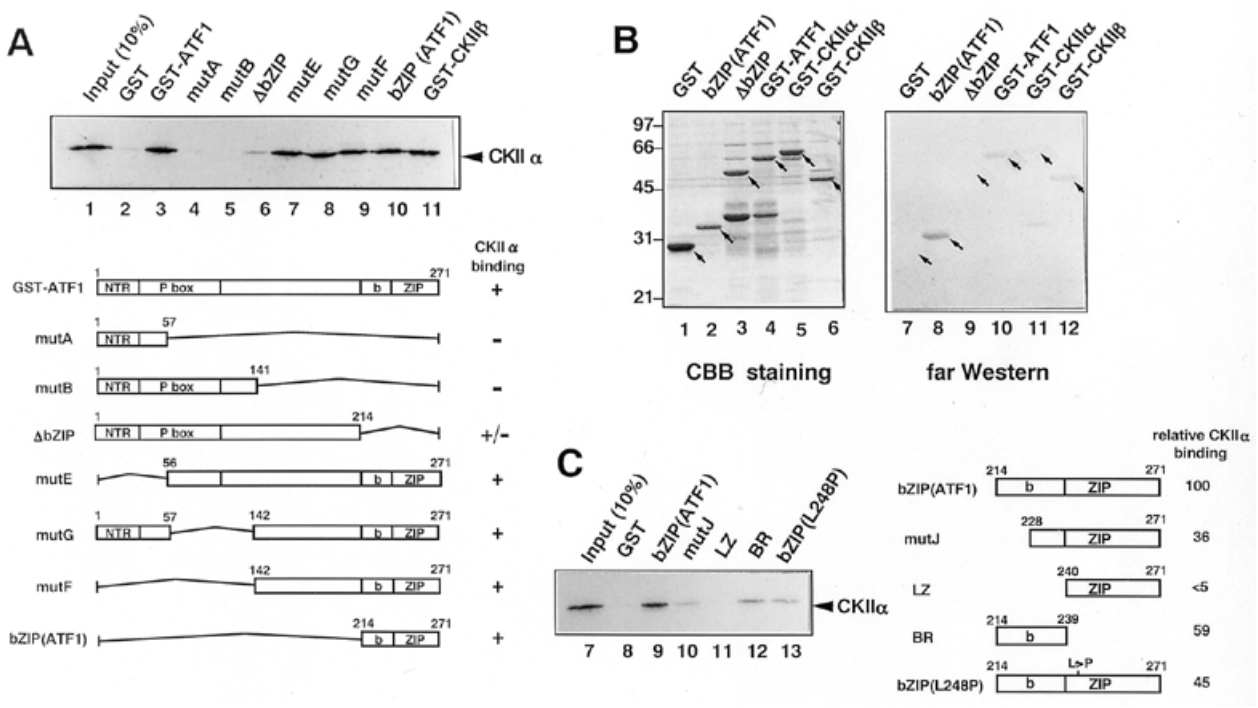

Figure 2. CKIIα binds the bZIP domain of ATF1. (**A**) The bZIP domain of ATF1 is necessary and sufficient for its interaction with CKIIα. Various ATF1 mutants fused to GST were expressed in *E.coli* and their ability to interact with *in vitro* translated CKIIα was examined as in Figure 1A. One microliter of the rabbit reticulocyte lysate containing ^{35}S-labeled CKIIα was used. (**B**) These interactions were analyzed by far-western blotting. Partially purified GST fusion proteins were resolved by SDS–PAGE and stained with Coomassie brilliant blue (lanes 1–6) or transferred to PVDF membrane. The blotted proteins were denatured, renatured and probed with ^{35}S-labeled CKIIα (lanes 7–12). (**C**) Mapping the region within the bZIP domain critical for its interaction with CKIIα. Various bZIP domain mutants fused to GST were constructed and examined for their ability to bind *in vitro* translated CKIIα. Binding of the wild-type bZIP to CKIIα (lane 9) is expressed as 100%.

Catalytically active CKII exists as heterotetramers, $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$ or $\alpha'_2\beta_2$ (3). This prompted us to ask whether CKII interacts with ATF1 as the holoenzyme. Bacterially expressed His-CKIIβ alone showed faint binding to GST-ATF1 (Fig. 1B, lane 12). However, when mixed with His-CKIIα to form the holoenzyme, His-CKIIβ bound GST-ATF1 to a level comparable with that of His-CKIIα (lane 13). The kinase activity of His-CKIIα was stimulated >10-fold by His-CKIIβ under these conditions (not shown), indicating the formation of catalytically active holoenzyme. These results demonstrate that holo-CKII also binds ATF1 and that CKIIβ indirectly interacts with ATF1 through CKIIα.

This interaction was also observed *in vivo* (Fig. 1C). FLAG-tagged ATF1 was transiently expressed in HeLa cells. The FLAG-ATF1-containing complex was immunoprecipitated with anti-FLAG monoclonal antibody and eluted with FLAG peptide. The presence of CKIIα in the eluate was examined by immunoblotting. CKIIα was detected only when FLAG-ATF1 was expressed (lane 4), indicating the interaction between FLAG-ATF1 and endogenous CKII within the cells. While FLAG-ATF1 was quantitatively recovered in the eluate, only a small fraction of CKII (a few percent) could be recovered. This suggests that CKII does not stably bind, but rather loosely associates with, ATF1 *in vivo* (see below).

CKIIα interacts with the bZIP domain of ATF1

We next mapped the region of ATF1 involved in binding to CKIIα. We constructed a series of ATF1 deletion mutants fused to GST and analyzed their ability to bind ^{35}S-labeled CKIIα synthesized *in vitro* (Fig. 2A). As CKIIβ binds CKIIα (20), GST-CKIIβ was used as a positive control (lane 11). N-Terminal or internal deletions of ATF1 did not affect its binding to CKIIα (lanes 7–9). Significantly, only the bZIP domain of ATF1 bound CKIIα at a similar level to that of the wild-type (lane 10). On the other hand, ATF1 mutants lacking the bZIP domain hardly bound CKIIα (lanes 4–6). We also examined their interactions with holo-CKII (Fig. 1B). The ATF1 bZIP domain bound holo-CKII (lanes 5–7), but the ATF1 mutant lacking the bZIP did not (lanes 8–10). Thus, we conclude that the bZIP domain of ATF1 is necessary and sufficient for its binding to CKII.

We confirmed the above results by far-western blotting (Fig. 2B). Several GST fusion proteins were partially purified by glutathione–Sepharose chromatography, resolved by SDS–PAGE and electrotransferred to a PVDF membrane. The immobilized proteins were renatured and probed with ^{35}S-labeled CKIIα. CKIIα detected GST-ATF1 and bZIP(ATF1) (lanes 8 and 10), but neither ΔbZIP (lane 9) nor control GST. These results are in good agreement with those of the GST pull-down assays (Fig. 2A).

To define the region of bZIP critical for its interaction with CKIIα more precisely, a series of bZIP derivatives was constructed and assayed as in Figure 2A (Fig. 2C). Deletion of half of the basic region diminished the interaction by 64% and complete deletion of this region abolished the interaction (lanes 4 and 5). On the other hand, a deletion of the leucine zipper or a point mutation that changed one of four conserved leucine residues to proline reduced the interaction moderately (lanes 6 and 7). Thus, the basic region of bZIP is essential for interaction with CKIIα, but the leucine zipper may also modulate binding. Since the point mutant L248P is unable to homodimerize (not shown), CKIIα may preferentially bind the bZIP dimer with higher affinity than the monomer.

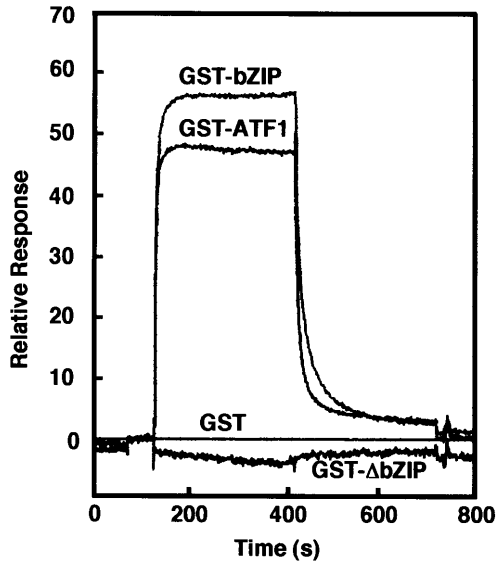

Figure 3. Kinetic analysis of CKII-ATF1 interaction. The interaction was measured with a SPR sensor system (BIAcore 2000), as described in Materials and Methods. One hundred and fifty microliters of 100 nM purified recombinant His-CKIIα was passed over the sensor surface containing 2180 RU GST-ATF1, 1750 RU GST-ΔbZIP or 1130 RU GST-bZIP (ATF1) at a flow rate of 30 µl/min. Shown are sensorgrams from which the response of the GST surface was subtracted. CKIIα shows significant binding to the GST-ATF1 and GST-bZIP surface, but not to the GST-ΔbZIP surface.

We also mapped the region of CKIIα involved in its interaction with the ATF1 bZIP. In summary, the N-terminal 84 amino acids of CKIIα is critical for this interaction (not shown). This region is highly conserved between CKIIα and CKIIα′ and contains a putative ATP-binding motif (3).

Kinetic analysis of the CKII-ATF1 interaction

We next sought to determine the kinetic parameters of the CKII-ATF1 interaction using a SPR sensor. GST-ATF1, its derivatives or control GST were immobilized on the sensor surface through the GST portion. Purified recombinant His-CKIIα was injected and passed over the sensor surface at a flow rate of 30 µl/min. The association and subsequent dissociation reactions were detected in real time and expressed as RU. Since the response of the sensor surface on which only GST was immobilized represents the bulk effect of the injected sample, the data presented in Figure 3 have the background subtracted. Under these conditions, CKIIα showed significant binding to the GST-ATF1 and GST-bZIP surfaces, but not to the GST-ΔbZIP surface, which is in good agreement with the above results. The kinetic constants of the CKII-GST-ATF1 interaction were calculated from the curve as described in Materials and Methods. The dissociation and association rate constants were $(4.6 \pm 0.8) \times 10^{-2}$/s and $(3.9 \pm 0.4) \times 10^{6}$/s/M and the equilibrium dissociation constant was $(1.2 \pm 0.3) \times 10^{-8}$ M.

The bZIP domain of ATF1 is required for its efficient phosphorylation by CKII

CKII phosphorylates Ser36 within the P box of ATF1 (21). As a first step towards understanding the function of CKII binding, we examined phosphorylation of the ATF1 mutants by CKII (Fig. 4). Neither mutE nor mutF, which lack Ser36, was phosphorylated

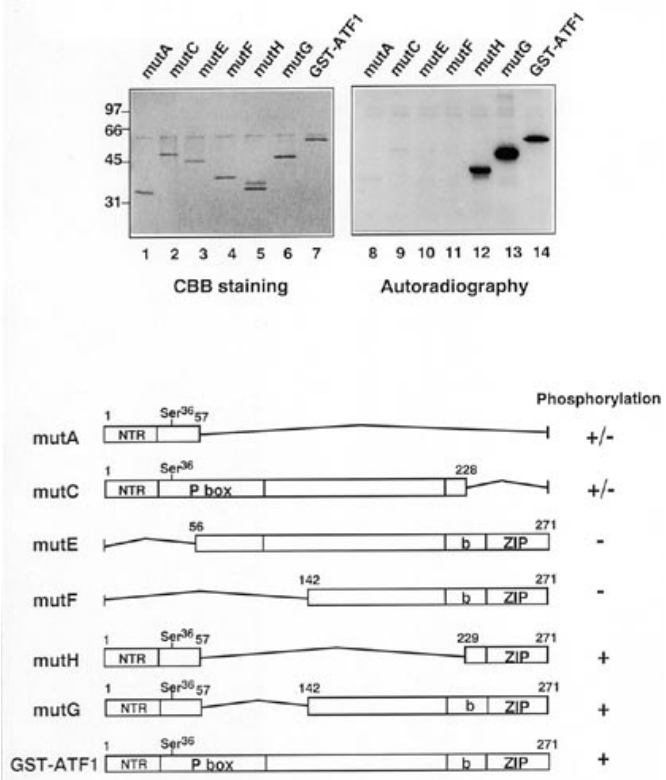

Figure 4. *In vitro* phosphorylation study of ATF1. Fifty nanograms of various ATF1 mutants were phosphorylated by 1 µl human CKII in 10 µl kinase buffer (50 mM KH_2PO_4/K_2PO_4, pH 7.2, 5 mM $MgCl_2$ and 100 mM KCl). Reactions were started by adding 10 µM ATP and 1 µCi [γ-^{32}P]ATP, incubated for 10 min at 30°C and stopped by adding SDS sample loading buffer. They were resolved by SDS–PAGE, stained with Coomassie brilliant blue (lanes 1–7) and detected by autoradiography (lanes 8–14). These results are summarized below. ATF1 contains the P box region at the N-terminus and Ser36 in this region is a primary target for phosphorylation by CKII (21).

(lanes 10 and 11), in agreement with a previous report (21). Wild-type ATF1 and the internal deletion mutants (mutG and mutH), which contain Ser36, were phosphorylated (lanes 12–14) efficiently. Interestingly, however, the C-terminal deletion mutants (mutA and mutC), which also contain Ser36, were only faintly phosphorylated (lanes 8 and 9). Note that comparable amounts of these mutants were used as substrates (lanes 1–7). This indicates that the C-terminal part of ATF1 is required for phosphorylation by CKII, in addition to the sequence around the phosphorylation site. Both mutA and mutC lack the C-terminal bZIP domain, whereas mutG and mutH have this domain (although the basic region is partly deleted in mutH). Combined with the results from the interaction analysis, it is likely that the presence of the bZIP domain enhances its phosphorylation, by recruiting CKII to the vicinity of the phosphorylation site.

ATF1-CKII complex can be formed on DNA

In a previous study, we identified CKII kinase activity in a fraction purified from HeLa nuclear extracts using DNA-affinity latex beads bearing ATF sites (19). Therefore, it might be possible that CKII binds DNA through ATF1. To address this issue, we examined binding of GST derivatives to a ^{32}P-labeled DNA fragment bearing an ATF site in the presence or absence of

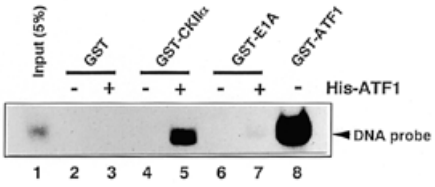

Figure 5. CKIIα binds ATF1 on DNA. A ^{32}P-labeled DNA fragment bearing an ATF site was incubated in the presence or absence of 100 ng bacterially expressed His-ATF1 for 30 min at 30°C. Binding of the DNA to CKIIα, E1A and ATF1 was examined as in Figure 1A. The bound DNA was separated by native PAGE and detected by autoradiography. Five percent of the input is shown in lane 1.

His-ATF1. As shown in Figure 5, GST-CKIIα bound the DNA fragment only in the presence of His-ATF1 (lanes 4 and 5). As a positive control, we employed GST-ATF1, which directly binds the DNA fragment (lane 8). As another control experiment, we used adenovirus E1A 13S protein, which reportedly binds ATF1 (22). GST-E1A also bound the DNA fragment in the presence of His-ATF1, but to a lesser extent than GST-CKIIα (lanes 6 and 7). From these results, we conclude that CKII forms a ternary complex with ATF1 and DNA. This finding would explain the molecular mechanism of CKII co-purification with the ATF family of transcription factors.

Gal4-CKIIα phosphorylates DNA-binding substrates via DNA

As demonstrated above, CKII can bind DNA through its interaction with the bZIP domain. To assess its potential effect, we developed a model experimental system. We constructed two chimeric proteins, Gal4-CKIIα and LexA-S (S for substrate; Fig. 6A). Gal4-CKIIα contains the catalytic α subunit of CKII fused to the DNA-binding domain of Gal4. LexA-S contains the most C-terminal octapeptide of the largest subunit of human pol II, which includes a phosphorylation site for CKII (23), fused to the DNA-binding domain of LexA. Gal4-CKIIα had similar catalytic activity to wild-type CKIIα and both phosphorylated LexA-S well (not shown). We also prepared DNA fragments containing Gal4, LexA or both sites (Fig. 6A) and examined their effects on the *in vitro* kinase reaction in which Gal4-CKIIα phosphorylates LexA-S (Fig. 6B). We established the conditions under which Gal4-CKIIα (1 nM) faintly phosphorylates LexA-S (1 nM) (lane 1). Under these conditions, galex, the DNA fragment containing both Gal4 and LexA binding sites, dramatically enhanced phosphorylation of LexA-S (lanes 11–13), whereas the other fragments did not (lanes 2–10). Significantly, the addition of two DNA fragments, gal and lex, had no effect (lanes 8–10). Therefore, these two binding sites must be located in the *cis* configuration to stimulate phosphorylation. These results suggest that enhanced phosphorylation is the consequence of recruiting both kinase and substrate onto the same DNA molecule and not due to their putative structural changes, which might be caused by binding to their specific binding sites.

When the DNA intercalater ethidium bromide (EtBr) was added to the reaction, DNA-mediated enhancement of phosphorylation was totally reversed (Fig. 6C, lanes 4–6). EtBr completely inhibited protein–DNA interaction at the indicated concentrations, as measured by the electrophoretic mobility shift assay (not shown), while it did not inhibit the basal kinase activity of Gal4-CKIIα

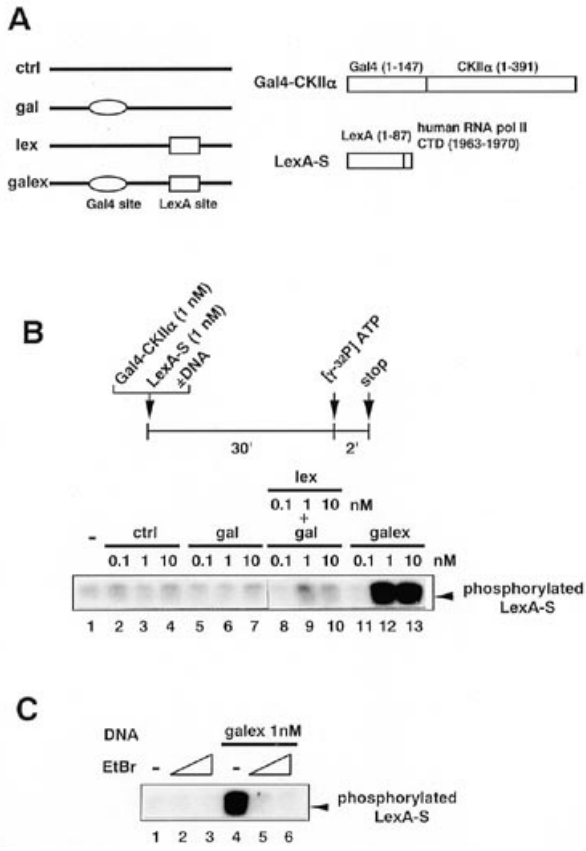

Figure 6. Gal4-CKIIα efficiently phosphorylates LexA-CTD via DNA. (**A**) A schematic representation of the DNA fragments (left) and chimeric proteins (right) used in (B) and (C). (**B**) Gal4-CKIIα (1 nM), LexA-CTD (1 nM) and the indicated concentrations of DNA fragment(s) were preincubated for 30 min at 30°C. Reactions were started by adding [γ-^{32}P]ATP, further incubated for 2 min at 30°C and stopped by adding SDS sample loading buffer. (**C**) Ethidium bromide (EtBr) was added to the preincubation reaction (lanes 2 and 5, 10 μg/ml; lanes 3 and 6, 100 μg/ml).

(lanes 1–3). Furthermore, when GST-S, which cannot bind DNA, was used instead of LexA-S, none of the DNA fragments affected phosphorylation (not shown). These two lines of evidence support the above interpretation.

CKIIα interacts with the bZIP domains of several transcription factors

The bZIP domain is highly conserved among the bZIP family of transcription factors (reviewed in 24). We therefore considered whether or not CKIIα interacts with the bZIP domains of transcription factors other than ATF1 by means of GST pull-down assays (Fig. 7A) and far-western blotting (Fig. 7B). In both assays, all of the bZIP domains tested (i.e. CREB, CRE-BP1, c-Fos and c-Jun) bound CKIIα (Fig. 7A, lanes 4–7, and Fig. 7B, lanes 9–12). This indicates that CKIIα recognizes amino acid residues conserved among the bZIP transcription factors.

DISCUSSION

In this study, we have demonstrated direct and specific interactions between protein kinase CKII and the bZIP transcription factors. We established that the α and α′ subunits of CKII directly bind ATF1, a member of the ATF/CREB family of transcription factors

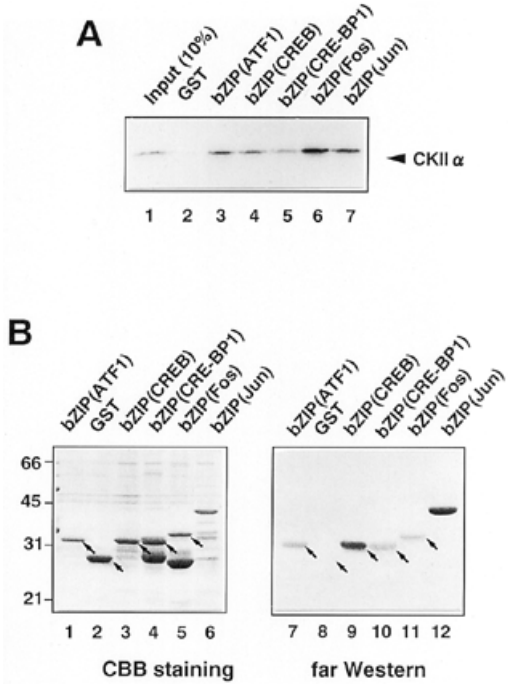

Figure 7. CKIIα interacts with the bZIP domains of several transcription factors. Different classes of DNA-binding domain were expressed as GST fusion proteins and their abilities to interact with CKIIα were examined by means of the GST pull-down assay (**A**) or far-western blotting (**B**). The partially purified proteins are shown in lanes 1–6 of (B).

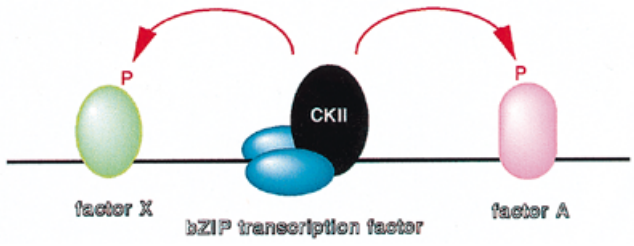

Figure 8. A possible model for transcription regulation by CKII.

(Fig. 1). CKII can indirectly associate with promoter DNA (Fig. 5) through this interaction. We previously co-purified CKII with ATF/CREB transcription factors using DNA-affinity latex beads bearing ATF sites (19). These results would explain the molecular basis for the co-purification. We assessed the equilibrium dissociation constant of the CKII-ATF1 interaction using an SPR sensor and obtained the value $(1.2 \pm 0.3) \times 10^{-8}$ M (Fig. 4). Considering the concentrations of CKII and ATF1 within the cells [~10^{-6} and ~10^{-8} M respectively, as determined by quantitative western blotting (not shown)], it is likely that CKII does not stably bind, but rather loosely associates with, ATF1 *in vivo*.

Mutational analysis of ATF1 revealed that the bZIP domain is necessary and sufficient for its interaction with CKIIα (Fig. 2A and B). CKIIα may preferentially bind the bZIP dimer with higher affinity than the monomer (Fig. 2C). Furthermore, CKIIα bound the bZIP domains of several transcription factors other than ATF1 (Fig. 7). This implies that CKII ubiquitously interacts with the bZIP transcription factors. We argue that this interaction should not occur simply due to electrostatic interactions with the basic residues in the bZIP domain, based on the following three points. First, the most N-terminal 84 amino acids of CKIIα, which is critical for bZIP binding (not shown), is not acidic, but rather basically charged (pI ~ 10.2). Second, a point mutation within the leucine zipper portion of the bZIP affects the strength of this interaction (Fig. 2C). Third, we performed alanine scanning mutagenesis within the basic region of the bZIP and found that the binding affinity does not necessarily correlate with the number of basic amino acids (not shown).

Many viral and cellular proteins, such as E1A 13S of adenovirus, Tax of human T cell leukemia virus, pX of hepatitis B virus and HMG I(Y), bind the bZIP domains of some transcription factors (22,25–27). These interactions are reportedly essential for their functions. For instance, E1A 13S can stimulate transcription of the adenoviral *E1B* gene by targeting the promoter DNA through its interaction with the bZIP domain of ATF2 (22). Similarly, CKII targets promoter DNA through its interaction with several bZIPs. The bZIP domain was originally identified as a DNA-binding and dimerization domain (28,29), but it is likely that this domain also functions as a protein–protein interaction surface that recruits heterologous proteins onto promoter DNA.

To elucidate the functional relevance of this interaction, we performed an *in vitro* phosphorylation study using ATF1 mutants as substrates. We found that deletion of the bZIP domain of ATF1 markedly reduces its phosphorylation (Fig. 5). In the primary sequence, the bZIP domain is located far from Ser36, a major phosphorylation site for CKII (21). This result can be interpreted as follows. First, the deletion may cause a structural change that makes Ser36 inaccessible to CKII. Second, the activity of CKII may be allosterically stimulated by binding to the bZIP domain. Third, the bZIP domain may enhance phosphorylation by recruiting CKII to the vicinity of its intramolecular phosphorylation site. The second model predicts that an excess amount of bZIP polypeptide stimulates CKII activity and the third model predicts the opposite result (by squelching the bZIP-interacting domain of CKIIα). Actually, it inhibited phosphorylation of ATF1 (21). Therefore, the third recruitment model is most likely, although we cannot exclude the first. There are several reports that protein kinases stably bind to their substrates (30–33). c-Jun N-terminal kinase (JNK), for example, interacts with its substrate c-Jun (32). The δ region of c-Jun, distinct from the phosphorylation sites, is required for both binding and phosphorylation by JNK.

We examined the consequence of recruiting CKII onto promoter DNA by using a model experimental system (Fig. 6). Phosphorylation of LexA-S by Gal4-CKIIα was greatly enhanced by the presence of a DNA fragment with binding sites for both. Several lines of evidence suggest that enhanced phosphorylation by the DNA fragment is the result of recruitment of both kinase and substrate in close proximity. It can also occur in the context of native promoter-containing binding sites for bZIP transcription factors. However, it would be difficult to test this using the system employed here, because the strength of the CKII-ATF1 interaction (K_d ~ 10^{-8} M) is not strong enough to localize CKII mostly on DNA, leading to overwhelming DNA-independent phosphorylation of the substrate. We do not imply from these experiments that the target of DNA-bound CKII is the pol II CTD or that the presence of specific DNA increases the total activity of CKII *in vivo*. What we suggest is that CKII, once recruited to promoter DNA, efficiently phosphorylates proteins bound to the nearby elements to alter their functions only at the time and place they work (see

model in Fig. 8). In this regard, it should be noted that many general transcription factors have a phosphorylation site(s) for CKII, although their physiological relevance is yet to be characterized.

To date, at least three protein kinases, DNA-dependent protein kinase (DNA-PK) (34), CDK-activating kinase (CAK) (35) and c-Abl (36), directly or indirectly bind DNA. From their unique properties, they are believed to regulate events occurring on DNA such as transcription, replication, recombination and repair by phosphorylating proteins on DNA. DNA-PK comprises a DNA-binding component termed Ku and a 460 kDa catalytic subunit, DNA-PKcs, and is activated by binding to double-stranded DNA ends (34,37). DNA-PK is supposed to induce the DNA repair process, possibly by phosphorylating the 34 kDa subunit of replication protein A (38,39). CAK, composed of MO15/CDK7, cyclin H and p36, is a kinase component of general transcription factor TFIIH (40–42). CAK indirectly binds promoter DNA as a preinitiation complex, together with the other general transcription factors, and phosphorylates the pol II CTD (35). The proto-oncogene product c-Abl is a tyrosine kinase that directly binds sequence-specific DNA through its unique DNA-binding domain (36). c-Abl stimulates transcription by potentiating the trans-activating function of some transcription factors (43). Here, we have identified another example of protein kinase that can associate with DNA. It will be of great importance to determine the role CKII plays on DNA.

In conclusion, we have demonstrated for the first time that protein kinase CKII interacts with bZIP domains of several transcription factors. This interaction leads to two consequences. First, CKII is recruited to the proximity of the intramolecular phosphorylation site (of ATF1) and efficiently phosphorylates it. Second, CKII indirectly binds promoter DNA and may phosphorylate proteins bound at the nearby element.

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