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Copurification of casein kinase II with transcription factor ATF/E4TF3

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ABSTRACT

We have developed a simple method to purify sequence-specific DNA-binding proteins directly from crude cell extracts by using DNA affinity latex beads. The method enabled us to purify not only DNA-binding proteins, but also their associated proteins. Using beads bearing the ATF/E4TF3 site from the adenovirus E4 gene promoter, a protein kinase activity was copurified with the ATF/E4TF3 family. We found that the kinase interacted with ATF1 *in vitro* efficiently. The kinase did not bind directly to DNA. The kinase mainly phosphorylated ATF1 on serine 36, which was one of target amino acids for casein kinase (CK) II. Biological features of the kinase were the same as those of CKII and an anti-CKII serum reacted with the kinase, indicating that the kinase was CKII. Moreover, it was clearly shown that one of CKII subunits, the CKII α protein bound to glutathione-*S*-transferase (GST) fusion ATF1 but not GST *in vitro*. It has been reported that a specific CKII inhibitor, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) inhibits transcription by RNA polymerase II [Zandomeni *et al.*, (1986) *J. Biol. Chem.* 261, 3414–3419]. Taken together, these results suggest that ATF/E4TF3 may recruit the CKII activity to a transcription initiation machinery and stimulate transcription.

INTRODUCTION

Generally, transcriptional activators are known to regulate the rate of initiation by interacting activators with general transcription factors (1–3). One of the strong transcription activators, Gal4-VP16, which is often used to investigate the transcriptional activation mechanisms, stimulates transcription dependent on the Gal4 binding site upstream of the transcription initiation site in the promoter region *in vivo* and *in vitro* (4–6). In this case, the transcription activation domain of VP16 binds to various factors in the preinitiation complex, transcription factor (TF)IID (7,8), TFIIB (9,10), TFIIH (11) and a coactivator, PC4 (3), and these interactions are known to be required for increasing the rate of initiation. However, Yankulov *et al*. have recently reported that transcription activators stimulate transcription by increasing not only the rate of initiation but also the processivity of RNA polymerase II (12). They have found that Gal4-VP16 and Gal4-E1a can stimulate transcription by enhancing processivity *in vivo*. Although the mechanisms remain unclear, the transcription activators can recruit elongation factors to the initiation complex. In these contexts, there is one reasonable interpretation that protein kinase activities are involved in the regulation of the processivity, since stimulation of processivity is specifically inhibited by one of the kinase inhibitors, the purine nucleoside analogue 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (13,14). For the reasons mentioned above, we proposed that some transcription activators could stimulate transcription by recruiting the kinase activities to enhance the processivity. It was reported that the transcription factor E2F, responsible for adenovirus E2A gene expression, associates with the cell cycle regulating factors, the retinoblastoma protein (pRb) and its related p107 protein, and pRb or p107 containing E2F complexes also contain cyclin E-cdk2 or cyclin A-cdk2 kinases (15 and references therein). These E2F complexes have sequence-specific DNA-binding activity, implying that E2F can recruit the protein kinase activities to the position in the vicinity of the preinitiation complex (16). In addition, as mentioned above, VP16 can also interact specifically with TFIIH which has kinase activities to phosphorylate the CTD (11,17). These data support our speculation that some activators have an ability to recruit the protein kinase activities to the initiation complex.

We have recently purified and characterized E4TF3, responsible for adenovirus E4 gene transcription, using DNA affinity latex beads (18). We showed that E4TF3 purified by the beads stimulated transcription *in vitro*, dependent on binding to E4TF3-binding sites.

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At least eight polypeptides with different molecular weights 116, 80, 65, 60, 55, 47, 45 and 43 kDa were copurified (18). The complex of polypeptides bound specifically to the E4TF3 sequence, implying that E4TF3 consists of a family of related factors. We now know that E4TF3 comprises the ATF/CREB family of proteins. Immunoblotting analysis showed that the 65, 47 and 43 kDa species are identical to ATF2/CRE-BP1, CREB and ATF1 respectively (unpublished data). It has been shown that the members of the ATF/CREB family are phosphorylated by several protein kinases and phosphorylation modulates the transcriptional activity. Casein kinase (CK) II (19,20), calmodulin/Ca^{2+}-dependent protein kinase (21,22), cAMP-dependent protein kinase (PKA) (23–26), protein kinase C (PKC) (26) and DNA-dependent protein kinase (DNA-PK) (20) have been reported to phosphorylate either ATF2/CRE-BP1, CREB or ATF1 at several sites *in vitro*.

In this paper, to analyze kinases associated with ATF/E4TF3, we performed the purification of ATF/E4TF3 by using the DNA affinity latex beads directly from HeLa cell nuclear extracts. Interestingly, we found the protein kinase activity involved in ATF/E4TF3. The kinase activity was able to phosphorylate ATF1 *in vitro* and retained on glutathione-*S*-transferase fusion (GST) ATF1 but not GST. Moreover, the kinase activity did not bind to latex beads bearing the ATF/E4TF3 site, implying that the kinase could not bind to DNA by itself. Surprisingly, the kinase activity had the same biological features as those of CKII, and the kinase fraction contained all subunits (α, α′ and β) of CKII (27), indicating an identity of the kinase with CKII. We found also that the CKII α protein actually bound to ATF1 *in vitro*. Zandomeni *et al*. have reported that CKII is involved directly or indirectly in the inhibition by DRB of specific RNA polymerase II-mediated transcription (28). Furthermore, DRB is a selective inhibitor of CKII (29). Therefore, we thought that ATF/E4TF3 can recruit the CKII activity to the initiation complex and this may regulate the transcriptional activity.

MATERIALS AND METHODS

Cell culture

HeLa spinner cells were grown in MEM containing 10% horse serum as described previously (30).

Preparation of HeLa cell nuclear extracts or whole cell extracts

Nuclear extracts were prepared according to the method of Dignam *et al*. (31). Whole cell extracts were prepared according to the method of Manley *et al*. (32).

Preparation of the DNA affinity latex beads

Latex beads carrying E4TF3 sites were prepared as described previously (18).

Construction of GST fusion protein expression vectors

The GST-ATF1 expression vector (pGEX-ATF1) was constructed by inserting a polymerase chain reaction (PCR)-generated fragment that contains ATF1 sequences from 190 to 1002 nucleotides into the plasmid pGEX-2T (Pharmacia) between *Sma*I and *Eco*RI sites (33).

The expression and purification of GST and GST fusion protein using glutathione–Sepharose were described previously (34).

Preparation of histidine tagged fusion proteins

The bacterial expression vectors and the purification of the histidine tagged fusion protein were described previously (20). Serine 36, 38 and 41 are changed to alanine. Each ATF1 mutant has a mutation(s) at the indicated position of serine (20).

Purification of protein kinase activity

Purification procedures were performed as described previously (18). Fifteen milliliters of HeLa cell nuclear extracts (8 mg/ml protein) were mixed with the latex beads bearing E4TF3 sites. After 30 min at 4°C, the latex beads were collected by centrifugation, and were washed with TGEN buffer [50 mM Tris (pH 8.0), 20% (w/v) glycerol, 1 mM EDTA, 1 mM dithiohreitol (DTT), 0.1% Nonidet-40 (NP-40)] containing 0.1 M KCl. Bound proteins were eluted with TGEN buffer containing 1.0 M KCl. After dialysis of the eluate against TGEN buffer containing 0.1 M KCl and 12.5 mM $MgCl_2$, the dialysate was loaded onto a Mono Q HR5/5 column (Pharmacia; 1 ml) at a flow rate of 1.0 ml/min. The column was washed with 20 ml TGEN buffer containing 0.1 M KCl and 12.5 mM $MgCl_2$. Proteins were eluted by 10 ml of a linear gradient of 0.1–0.5 M KCl. Fractions were collected every 30 s. Fractions containing activity from the Mono Q step were pooled and aliquots (150 µl) were mixed with 50 µl TGMN buffer [50 mM Tris (pH 7.9), 1 mM EDTA, 12.5 mM $MgCl_2$, 1 mM DTT, 0.1% NP-40]. The diluted sample was loaded on 4.8 ml 15–35% glycerol gradient. After centrifugation using SW 50.1 rotor (Beckman) at 48 000 r.p.m. for 24 h at 4°C, gradient was divided into 25 fractions. These fractions were analyzed by the protein phosphorylation assay. Fractions 18–24 were pooled and used as the kinase fraction.

Protein phosphorylation assay

Phosphorylation of protein was performed at 30°C in a reaction mixture (10 µl) containing 50 mM KH_2PO_4–K_2HPO_4 (pH 7.2), 10 mM $MgCl_2$, 3–5% glycerol, 100 mM KCl and 100 µl ATP containing 1 µCi [γ-^{32}P]ATP (3000 Ci/mmol). The reaction was terminated by the addition of 5 µl 4× Laemmli sample buffer and boiled for 5 min. Proteins were subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and stained by Coomassie brilliant blue and the gel was dried for autoradiography. During purification, protein kinase activity was monitored with GST-ATF1 (0.05 mg/ml) as the substrate.

Quantitation of phosphate incorporation was determined with an AMBIS radioanalytic system.

Protein kinase A catalytic subunit was purchased from Sigma Chemical Co.

CKII purified from pig testes was kindly provided by Dr Nakaya (35).

Phosphoamino acid analysis of GST-ATF1

Two kinases, PKA (0.1 U) and the kinase fraction (1 µl) were used for phosphorylation of GST-ATF1 respectively. ^{32}P-labeled GST-ATF1 (200 ng) was fractionated on a 10% SDS polyacrylamide gel, and the gel containing the labeled GST-ATF1 was excised and washed vigorously by shaking in 500 µl of 25% 2-propanol for 6 h. Then the gel was washed in 500 µl 10% methanol for 2 h on a rotator. After drying the gel, 50 µl HCl was added and incubated at 110°C in an oil bath for 2 h (36). The solution was lyophilyzed and resuspended in 5 µl H_2O. Aliquots (2 µl) of sample were mixed with a phosphoamino acid standard, then subjected to thin layer

electrophoresis using Pharmacia Multiphor II Electrophoresis system. After drying, standards were visualized by spraying with 5% ninhydrine in methanol and incubated at 65°C for 5 min. Radiolabeled amino acids were detected by autoradiography.

In vitro binding assay

Glutathione–Sepharose beads (25 µl) that had been incubated with bacterial supernatant fractions containing GST (25 µl) and GST-ATF1 (125 µl) were washed three times with NETN buffer [20 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% NP-40] and once with TGEN buffer containing ~1 mg/ml immobilized GST and GST-ATF1. Twenty five microliters of the kinase fraction was diluted with an equal volume of TEMN buffer. The diluted samples were mixed with 25 µl of GST-coupled beads to remove non-specific binding proteins, and the flow-through fractions were collected. The beads bearing immobilized GST and GST-ATF1 were incubated with aliquots (15 µl) of the flow-through fraction at 4°C for 60 min and were washed five times with TGEN buffer containing 0.1 M KCl, before bound proteins were eluted with 25 µl TGEN buffer containing 1.0 M KCl and 12.5 mM $MgCl_2$. The ATF1 kinase activity of each sample was analyzed.

^{35}S-labeled CKII α protein was synthesized *in vitro* by transcription of CKII α cDNA (pET-CKII α) by using T7 RNA polymerase (Takara) and subsequent translation in rabbit reticulocyte lysate basically according to the manufacturer's instructions (Promega). The CKII α expression vector, pET-CKII α, was constructed by inserting a PCR-generated fragment that contains human CKII α sequences from 157 to 1332 nucleotides (37) into the plasmid pET14b between *Nde*I and *Bam*HI sites. ^{35}S-labeled CKII α protein was incubated with beads (25 µl) bearing ~1 mg/ml immobilized GST and GST-ATF1 for 60 min at 4°C. After washing beads, bound proteins were eluted by boiling in 1× SDS sample dye and subjected to SDS–PAGE and autoradiographed.

Purification of CKII

CKII was purified from whole cell extracts prepared as described by Manley *et al.* (32). Forty milliliters of the extracts (10 mg/ml proteins) were loaded onto a 40 ml heparin–agarose column equilibrated with HGEN buffer (20 mM Hepes, 20% glycerol, 1 mM EDTA, 1 mM DTT, 0.1% NP-40) containing 0.1 M KCl and 12.5 mM $MgCl_2$. Bound proteins were eluted stepwise as described previously (30). The E4TF3 DNA-binding activity was completely eluted at 0.4 M KCl (30). The CKII activity was eluted at 1.0 M KCl. The active fractions were pooled and proteins were precipitated by ammonium sulfate (final 40%). Then the precipitate was resuspended with 2 ml HG(10)EN (HGEN except 10% glycerol) buffer containing 0.1 M KCl and 10 mM $MgCl_2$ and loaded onto a Superdex 200 HR (10/30) column (Pharmacia) at a flow rate of 0.5 ml/min. The CKII activity was eluted from 7 to 13 ml after injection. The active fractions were pooled and loaded on a Mono SPC16/5 column (Pharmacia; 0.1 ml) equilibrated with HG(10)EN buffer containing 0.1 M KCl and 10 mM $MgCl_2$ at a flow rate of 0.1 ml/min by using the SMART System (Pharmacia). Proteins were eluted by 2.2 ml of a linear gradient of 0.1–0.5 M KCl. The CKII activity was recovered separately in flow-through fractions and gradient fractions eluted between 0.22 and 0.28 M KCl. The gradient fractions were pooled and dialyzed against TGEN buffer containing 0.1 M KCl and 10 mM $MgCl_2$. The flow-through fractions and the dialysate were loaded independently onto a Mono QPC16/5 column (Pharmacia, 0.1 ml) at a flow rate of 0.1 ml/min and proteins were eluted by 2.2 ml of a linear gradient of 0.1–0.5 M KCl. In both cases, the CKII activity was eluted at the same KCl (0.29 M) and used for further analysis as HeLa cell CKII.

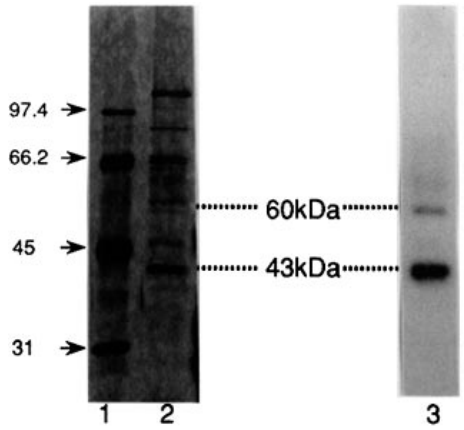

Figure 1. Protein kinase activity in the E4TF3 fraction. Aliquots (10 µl) of the E4TF3 fraction were subjected to SDS–PAGE and proteins were stained by silver (lane 2). Lane 1 contained standard protein makers: 97.4 kDa, phosphorylase B; 66.2 kDa, bovine serum albumin; 45 kDa, ovalbumin; 31 kDa, carbonic anhydrase. Two microliters of the E4TF3 fraction was mixed with [γ-^{32}P]ATP in a kinase reaction and incubated for 30 min at 30°C. The mixture was subjected to SDS–PAGE and the gel was dried and autoradiographed (lane 3). The gel was exposed with an intensifying screen for 1 week.

Immunoblotting assay

Immunoblotting was carried out as described previously (30). Proteins were subjected to SDS–PAGE and the fractionated proteins were transferred to Immobilon (Millipore) by wet electrophoretic transfer. After blocking the membrane with blots, the membrane was soaked with 500-fold diluted anti-CKII rabbit serum kindly provided by Dr Kikkawa (38). Then the membrane was developed according to the manufacturer's instructions (Amersham).

RESULTS

Detection of protein kinase activity in affinity-purified ATF/E4TF3

We purified the ATF/E4TF3 family of proteins directly from HeLa cell nuclear extracts by using DNA affinity latex beads having ATF/E4TF3 binding sites (18). To examine whether the purified fraction contained protein kinase activities, we carried out protein phosphorylation assay by adding [γ-^{32}P]ATP to aliquots of the fraction. As shown in Figure 1, we observed two labeled bands (of 43 and 60 kDa) on SDS–PAGE after incubation, indicating the presence of a protein kinase in the affinity-purified E4TF3 fraction. The affinity-purified fraction contained at least eight distinct polypeptides as described previously (Fig. 1, lane 2) (18). Interestingly, one of the phosphorylated proteins seemed to correspond to the 43 kDa protein known to be ATF1. We have not yet characterized the 60 kDa protein. This result suggested that the copurified kinase activity with the ATF/E4TF3 family probably phosphorylated the 43 kDa protein.

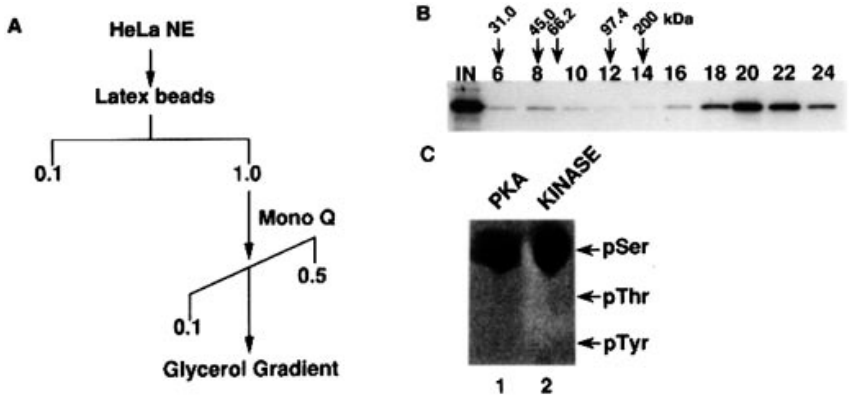

Figure 2. Analysis of fractions after sedimentation velocity centrifugation. (**A**) Schematic representation of the chromatographic scheme used to purify the kinase activity from HeLa nuclear extracts. The numbers given are KCl molarities. (**B**) Fractions containing activity in the Mono Q step were pooled and aliquots (150µl) were mixed with 50 µl TGMN buffer. The diluted sample was loaded onto 4.8 ml of a 15–35% glycerol gradient. The gradient was divided into 25 fractions. Numbers on the top indicate the positions of the standard proteins (as for Fig. 1). Reactions containing 1µl of each fraction and [γ-^{32}P]ATP were incubated at 30°C for 10 min using GST-ATF1 as the substrate. After incubation, samples were subjected to SDS–PAGE and the gel was dried and autoradiographed. (**C**) Phosphoamino acid analysis. [^{32}P]phosphate-labeled GST-ATF1 (1 µg) by PKA (lane 1) or the kinase fraction (lane 2) were separated on a 10% SDS polyacrylamide gel and the gel was analyzed using an AMBIS imaging analyzer. A piece of the gel containing the labeled GST-ATF1 was excised and subjected to phosphoamino acid analysis. Radiolabeled phosphorylated amino acids were detected by autoradiography.

Characterization of protein kinase activity

We usually washed the beads five times with buffer containing 0.1 M KCl to purify E4TF3 from nuclear extracts (18), although the washing step reduced a yield of the kinase activity (data not shown). Competitors [single strand DNA and poly(dI–dC):poly(dI–dC)], were also omitted, because they also reduced the activity (data not shown). The purification of the kinase activity was monitored using GST-ATF1 as the substrate in the protein phosphorylation assay. The kinase activity phosphorylated efficiently GST-ATF1 protein but not GST protein (data not shown).

Next, the eluate from beads was dialyzed and loaded on a Mono Q column. Protein kinase activity was eluted with a 10 ml linear gradient of 0.1–0.5 M KCl (Fig. 2). The ATF1 kinase activity peak eluted between 0.22 and 0.28 M KCl; most of the DNA binding activity, however, was detected in the flow-through fractions (data not shown), suggesting that the kinase loosely associated with ATF/E4TF3.

We purified the kinase activity further by sedimentation velocity centrifugation using glycerol. Fractions containing activity from the Mono Q step were pooled and aliquots were loaded on a 15–35% glycerol gradient. After the centrifugation, the gradient was divided into 25 fractions and analyzed using the protein phosphorylation assay (Fig. 2A and B). A peak of activity (fractions 18–24) appears to contain proteins of >200 kDa. Fractions 18–24 were pooled and used as the kinase fraction for further studies. Phosphoamino acid analysis was also performed using GST-ATF1 phosphorylated by the kinase activity (see Materials and Methods). After hydrolysis, the products were separated on a thin layer chromatography (TLC) plate and the phosphoamino acids, phospho serine, phospho threonine and phospho tyrosine were visualized by ninhydrine. The TLC plate was then autoradiographed. Figure 2C shows that the target amino acid of the kinase was a serine residue. Lane 1 is a control using GST-ATF1 phosphorylated by PKA. PKA phosphorylated a serine residue in the P box of ATF1 as reported previously (23).

Association of the kinase activity with ATF1

The kinase activity was isolated from the E4TF3 fraction purified using beads, presumably through an ability to interact with members of the ATF/E4TF3 family. To confirm the interaction *in vitro*, the kinase fraction was incubated separately with GST and GST-ATF1. After extensive washing, the bound proteins were released from the beads by the addition of high salt. The input samples and materials that eluted from the beads were subsequently assayed for ATF1 kinase activity and the products were analyzed by SDS–PAGE and autoradiography. Figure 3A shows that the kinase activity was selectively retained on GST-ATF1, but not on GST columns, indicating that the kinase activity associated with ATF1.

We also examined whether the kinase is able to bind directly to DNA and/or latex beads, independently of other proteins such as ATF1. To test this, the kinase fraction was mixed with DNA latex beads bearing the E4TF3 binding site. The beads were then washed extensively and the bound material was eluted and assayed for ATF1 kinase activity. As a comparison, crude nuclear extracts containing ATF/E4TF3 were processed in parallel. As shown in Figure 3B, the kinase activity did not bind directly to DNA latex beads and ATF kinase activity was only recovered in eluates from complete crude nuclear extracts. This implies, therefore, that the kinase activity was associated with at least ATF1.

Similarities between the kinase activity and CKII

Recently, Masson *et al*. have demonstrated that the critical serine residues map to a putative transcription activation domain of ATF1 using various ATF1 mutants (20). They showed that serine 36, 38 and 41 were involved *in vitro* phosphorylation of ATF1. Therefore, we used some of the ATF1 mutants to identify the site(s) of phosphorylation catalyzed by the kinase activity. Histidine tagged fusion ATF1 (H6-ATF1) and mutants proteins were isolated (20) and incubated with the kinase fraction (Fig. 4A). Changing serine 36 to alanine (Mut 36) caused a reduction of the susceptibility of the kinase activity and Mut 36/38 showed a greatly reduced

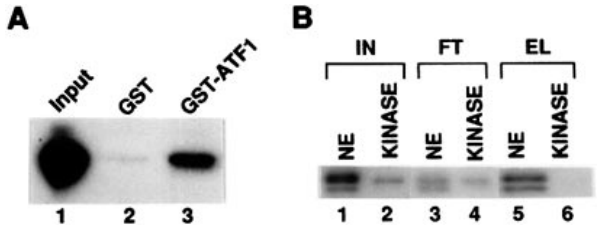

Figure 3. Protein kinase activity binds to ATF1. (**A**) Aliquots (50 µl) of the kinase fraction were diluted and chromatographed on 25 µl affinity column containing GST protein. Aliquots (25 µl) of the flow-through fraction were rechromatographed on 25 µl of each affinity column indicated. After washing the column, bound protein was eluted with TGEN buffer containing 1.0 M KCl and 12.5 mM $MgCl_2$. The diluted input activity (Input, lanes 1: 0.1 µl) and the eluate fractions (lanes 2 and 3: 2 µl) were analyzed using the GST-ATF1 kinase assay. (**B**) NE; 0.2 µl nuclear extracts in 100 µl TGEN buffer containing 0.1 M KCl, KINASE; 100 µl of the kinase fraction for starting materials. Samples were mixed well with the latex beads bearing 0.5 µg E4TF3 DNA-binding sites, and the beads were incubated at 4°C for 30 min. After centrifugation, the flow-through fractions (FT) were collected, and the beads were washed five times with 100 µl TGEN buffer containing 0.1 M KCl, and then bound proteins were eluted with 10 µl TGEN buffer containing 1.0 M KCl. One microliter of each material (lane 1, NE; lane 2, the kinase fraction) or flow-through (lane 3, NE; lane 4, the kinase fraction) or eluate (lane 5, NE; lane 6, the kinase fraction) was tested for ATF1 kinase activity.

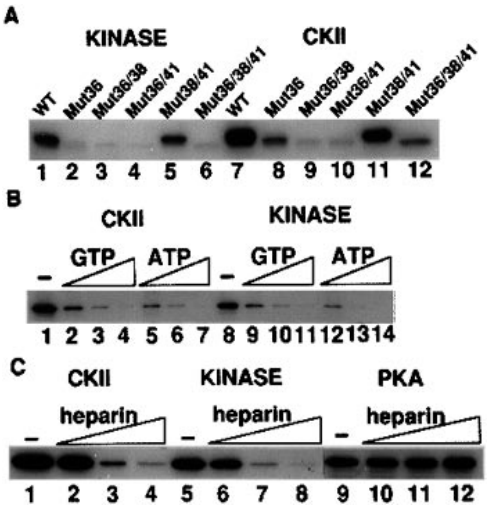

Figure 4. Similarities between the kinase and CKII. (**A**) Substrate proteins indicated were added at 50 µg/ml to a phosphorylation reaction containing either the kinase fraction [1 µl or pig CKII (5 ng)]. After 10 min incubation, samples were subjected to SDS–PAGE and the gel was dried and autoradiographed. (**B**) Reactions containing GST-ATF1 (50 µg/ml) and either pig CKII (5 ng, lanes 1–7) or the kinase fraction (1 µl, lanes 8–14) were incubated with 10 µM [γ-^{32}P]ATP at 30°C (lanes 1 and 8), plus GTP or ATP as cold competitor (39 µM, lanes 2, 5, 9 and 12; 90 µM, lanes 3, 6, 10 and 13; 270 µM, lanes 4, 7, 11 and 14). (**C**) Specific inhibition of the kinase activity by low concentration of heparin. Reactions containing 0.5 µg GST-ATF1 and pig CKII (5 ng, lanes 1–4), the kinase fraction (1 µl, lanes 5–8), PKA (0.2 U, lanes 9–12) were incubated with or without heparin. Lanes 2, 6, 10 and 14 contain 10^{-4} U heparin; lanes 3, 7, 11 and 15 contain 5×10^{-4} U heparin; lanes 4, 8, 12 and 16 contain 10^{-3} U heparin.

susceptibility of the kinase activity compared with Mut 36. However, the kinase activity efficiently phosphorylated Mut 38/41 as well as wild-type ATF1, strongly suggesting that the major phosphorylation site of the kinase activity was serine 36 in the P box. We also observed that phosphorylations decreased the mobility of ATF1 in SDS gels (Fig. 4A). This was also represented by using *in vitro* translation products of ATF1 mRNA in rabbit reticulocyte lysate (20). Significantly, mutation of serine 36 alone had a dramatic effect on mobility (Fig. 4A), and double mutants Mut 36/38 and Mut 36/41 both gave a greater effect as compared with Mut 36. Interestingly, the mobility of Mut 38/41 on SDS gels was similar to that of ATF1, suggesting that serine 36 is most important for phosphorylation of the kinase activity and a phosphorylation-induced conformational change in ATF1. Furthermore, serine 36 which is lacking in CREB and CREM, is known to be phosphorylated by CKII *in vitro* as reported previously (20). To compare the phosphorylation states of these mutants, therefore, we used CKII purified from pig testes (35). As seen in Figure 4A, phosphorylation states of mutants by pig CKII were almost the same as that of mutants by the kinase activity. This result suggests that phosphorylation site sequences are highly homologous between the kinase activity and CKII.

We examined further other biological characters of the kinase activity with those of CKII. The CKII activity is capable of using either ATP or GTP as the substrate with similar K_m values and can be inhibited with low concentration of polyanionic compounds, such as heparin. To test whether the kinase activity can use GTP, protein phosphorylation assays were done in the presence of excess amount of cold GTP as competitor. As seen in Figure 4B, excess amount of cold GTP as well as ATP reduced the incorporation of ^{32}P to GST-ATF1 by both kinases in a dose-dependent manner, indicating that the kinase activity could use GTP instead of ATP in the same way as CKII. Furthermore, specific inhibition of the kinase activity by heparin is shown in Figure 4C. The activities of both CKII and the kinase were inhibited by at least 5×10^{-4} U heparin but PKA was not. These results suggested that the kinase associated with ATF1 was CKII. To confirm this, we purified CKII from HeLa cell extracts using a conventional column chromatography method.

The kinase fraction contains subunits of CKII

CKII was monitored during several successive chromatography steps, as outlined in Figure 5A, by immunoblotting and protein phosphorylation assays. After gel filtration, the CKII fractions were pooled and loaded onto a Mono S column using the SMART system. The CKII activity was observed in two different peaks, in flow-through fractions and gradient fractions which eluted between 0.25 and 0.3 M KCl (Fig. 5C) (see Discussion). Both active fractions were independently loaded on a Mono Q column and proteins were eluted by a linear gradient. Surprisingly, in both cases, the CKII activity was eluted in the same fraction number (Fig. 5D and E). Proteins included in the fractions were subjected to SDS–PAGE, and stained by silver (Fig. 5B, lanes 1 and 2). Subunits of CKII were detected in the fractions by an immunobotting assay (Fig. 5B, lanes 3 and 4). Three bands with molecular weights of 44, 40 and 28 kDa were observed mainly by silver staining, and these bands reacted specifically with the anti-CKII polyclonal antibody, indicating that the final fractions contained α, α′ and β subunits of CKII with molecular weight of 44, 40 and 28 kDa respectively (27). Therefore, we used the fraction as HeLa cell CKII. To confirm the HeLa cell CKII activity, we performed the same experiments as described in Figure 4. The HeLa cell CKII activity specifically inhibited by a low concentration of heparin and could use GTP as a phosphate donor. Furthermore, phosphoryla-

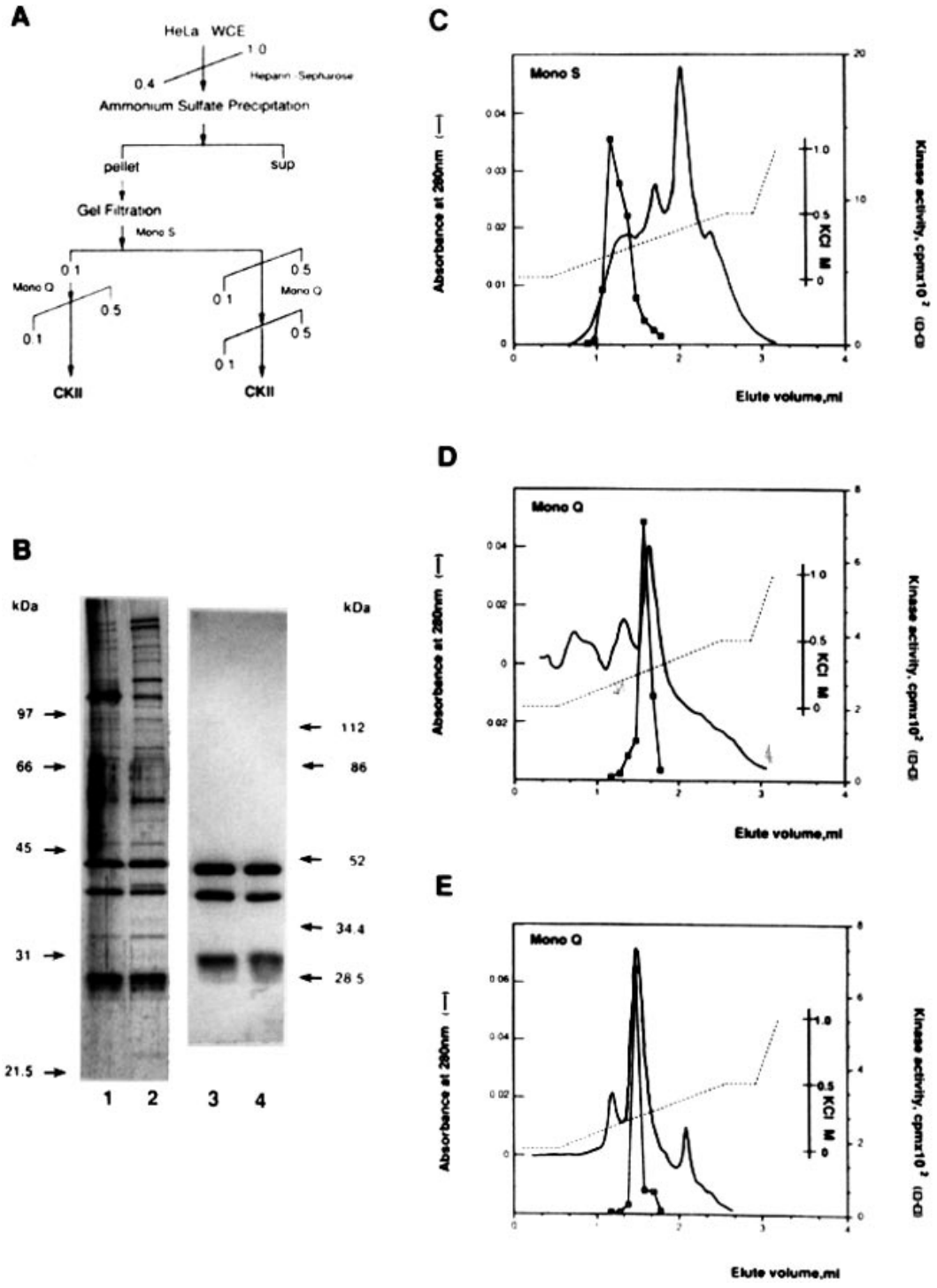

Figure 5. Purification of CKII from HeLa cell extracts. (**A**) Schematic representation of the chromatographic scheme used to purify CKII from HeLa cell extracts. The numbers given are KCl moralities. (**B**) The flow-through and gradient fractions at the Mono S step were independently loaded onto the Mono Q column as described in (D) and (E). The active fractions derived from the flow-through (lanes 1 and 3: 3µl) or the gradient (lanes 2 and 4: 2 µl) were subjected to SDS–PAGE and proteins were stained by silver (lanes 1 and 2) or immunoblotted by the anti-CKII polyclonal antibody (lanes 3 and 4). Numbers at the left side indicate the positions of the standard proteins as described in Figure 1 and at the right side indicate the positions of the prestained proteins, 112 kDa, phosphorylase B; 86 kDa, bovine serum albumin; 52 kDa, ovalbumin; 34.4 kDa, carbonic anhydrase; 28.5 kDa, soybean trypsin inhibitor. (**C**) Mono S chromatography. The active fractions from the Superdex column were loaded onto the Mono S column and proteins were eluted by a linear gradient of 0.1 M KCl and 0.5 M KCl. (—) Absorbance at 280 nm, (-□-) CKII activity, (- - - -) KCl concentration. (**D**) and (**E**) Mono Q chromatography. The flow-through fractions (D) or gradient fractions (E) between 0.22 and 0.28 were loaded onto the Mono Q column and proteins were eluted by a linear gradient of 0.1 and 0.5 M KCl. (—) Absorbance at 280 nm, (-□-) CKII activity, (- - - -) KCl concentration.

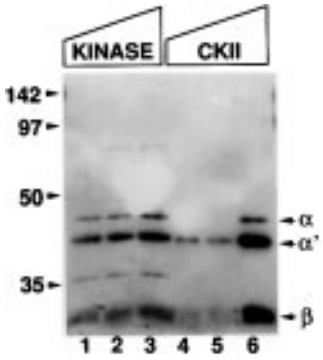

Figure 6. Identity of the kinase with CKII. Various amount of the kinase fraction (lane 1, 0.2 µl; lane 2, 0.25 µl; lane 3, 0.33 µl) and HeLa cell CKII (lane 4, 0.4 µl; lane 5, 0.5 µl; lane 6, 0.6 µl) were subjected to SDS–PAGE and separated proteins were transferred to membrane and immunoblotted by the anti-CKII polyclonal antibody. Numbers at the left side indicate the position of the standard protein size marker, and α, α′ and β indicate the positions of subunits of CKII respectively.

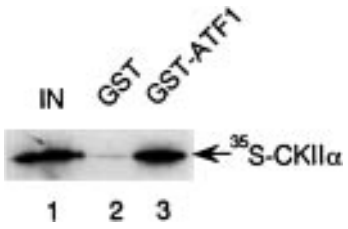

Figure 7. Binding of the CKII α subunit to ATF1 *in vitro*. ^{35}S-labeled CKII α protein was synthesized *in vitro* using reticulocyte lysate as described in Materials and Methods, and mixed with beads bearing immobilized GST and GST-ATF. After washing of the beads, bound proteins were eluted and subjected to SDS–PAGE. Then the gel was dried and autoradiographed. Lane 1, 10% of the input; lane 2, the eluate from the GST column; lane 3, the eluate from the GST-ATF1 column.

tion states of ATF1 mutants by HeLa cell CKII were completely the same as those of mutants by pig CKII (data not shown).

We further carried out an immunoblotting assay using the anti-CKII polyclonal antibody (38). As seen in Figure 6, the kinase fraction contained α, α′ and β subunits of CKII (lanes 1 and 3) as observed in HeLa cell CKII (lanes 4 and 5), indicating an identity of the kinase with CKII. The ratio of α:α′ was different from the result in Figure 5B. We think that this discrepancy was probably caused by different detection methods: we used a color detection method using horse radish peroxydase and diaminobenzine–HCl in Figure 5, but an ECL method was performed in Figure 6. However, distribution of all subunits of CKII are completely the same between HeLa cell CKII and the kinase fraction. These results showed an identity of the kinase to CKII.

To confirm the interaction between ATF/E4TF3 and CKII, we carried out the GST pull-down assay using CKII α subunit protein which was translated *in vitro* in the presence of [^{35}S]methionine using reticulocyte lysates (Promega). The ^{35}S-labeled CKII α protein was incubated separately with GST and GST-ATF1. After extensive washing, the bound proteins were released from the beads by boiling in the presence of the SDS–PAGE sample dye, the input sample and materials that eluted from the beads were analyzed by SDS–PAGE and autoradiography. As seen in Figure 7, the CKII α protein specifically bound to GST-ATF1 but not GST, indicating the physical interaction of ATF1 with CKII α protein *in vitro*. We also detected the CKII activity in the immunoprecipitate of HeLa cell extracts by anti-ATF1 serum but not anti-E1A serum which was used as a control material (data not shown). These results suggest that copurification of the CKII activity was due to the interaction of members in the ATF/E4TF3 family with the subunit of CKII and we proposed that ATF/E4TF3 can recruit CKII and may partly regulate transcription by CKII activity (see Discussion). It has been reported that DRB which is a specific inhibitor of the CKII activity specifically inhibits RNA polymerase II-driven transcription and the inhibition was partially resumed by addition of purified CKII (28). Therefore, we thought a possible involvement of action of CKII in transcription.

DISCUSSION

We have found a protein kinase activity in highly purified ATF/E4TF3 samples capable of phosphorylating the 43 kDa protein, ATF1 which is a member of the ATF/E4TF3 family (33). The kinase activity does not have a DNA binding activity but can interact with ATF1, and this specific interaction leads to copurification of the kinase activity with the ATF/E4TF3 family when ATF/E4TF3 was purified by using the DNA affinity latex beads. The kinase was CKII and one of the CKII subunits, CKII α, bound to ATF1 *in vitro*, suggesting a possible involvement of CKII in transcription.

CKII interacts with a variety of cellular factors

In this study, we purified CKII partially from HeLa cell extracts. At the step of the Mono S column, the CKII activity was divided into two parts, flow-through and gradient elution fractions (Fig. 5). CKII is known to associate with a variety of cellular proteins, topoisomerase I, topoisomerase II, p53, Hsp 70, nuclear matrix proteins and unknown nuclear proteins (39 and references therein). These associations probably caused the separation of the CKII activity. Actually, the CKII activity was recovered in fractions containing proteins ≤ 200 kDa molecular weight range by velocity sedimentation centrifugation (Fig. 2). It has been reported that the TFIID activity also separately eluted from a phosphocellulose column by the difference of components of TFIID (40).

Interaction of CKII with ATF1

Various protein kinases including CKII, PKA and DNA-PK are known to phosphorylate ATF1 *in vitro* (20,33). No direct interaction between the kinases and ATF1 has been reported. Interestingly, our identified protein kinase, CKII, could associate with ATF1 *in vitro* (Fig. 7) and *in vivo* (data not shown). Recently, we have analyzed the interaction between CKII and ATF1 by using GST pull-down assay and Far Western blotting. These analyses demonstrated that the catalytic α subunit of CKII directly interacts with ATF1, ATF2 and CREB (41). Furthermore, this interaction is functionally important for ATF1, since CKII-mediated phosphorylation of ATF1 was dependent on the binding of CKII to ATF1. However, other members of the ATF/E4TF3 family, ATF2 and CREB, were not efficiently phosphorylated by CKII even though CKII bound directly to them (data not shown).

Effects of CKII on transcription

Recently, several studies have reported effects of the CKII activity on transcriptional regulation. It has been shown that the specific function of anti-oncogene product, p53, is to work as a transcription factor (42–44). Interestingly, the two proteins, CKII and p53, associated in a molecular complex (45), suggesting that p53 can recruit the CKII activity to the initiation complex. On the other hand, topoisomerase I, which is also involved in the regulation of transcriptional activity, specifically binds to CKII (46,47), and CKII is likely to regulate function of topoisomerase I (47). It is known that topoisomerase I is included in the TFIID complex (48). In addition, Robitzki *et al*. have reported that endogeneous β subunit of CKII was increased when the α subunit of CKII was overexpressed by transfection in HeLa cells and the CKII α subunit appeared to bind specifically to the element in the CKII β gene promoter (49). They speculated that CKII α had an ability to stimulate transcription depending on its binding element. Taken together, these results strongly suggested that action of CKII is directly or indirectly involved in transcription and we thought that ATF1 may serve as a scaffold for CKII which can phosphorylate proteins in the vicinity of the ATF1 site and these phosphorylations may function in transcription. Actually, the target sequence of CKII is observed among several proteins in general transcription factors, TFIIA α, TFIIE, the large subunit of TFIIF and the largest subunit of RNA polymerase II (50). Interestingly, DRB inhibits RNA polymerase II-driven transcription specifically (28,51,52). *In vitro* experiments have shown a close correlation between the DRB-sensitivity of CKII and transcription (28). Recent studies have also shown that transcriptional activators affected the efficiency of elongation in transcription (12) and this activity specifically inhibited by DRB (13,14), however, the mechanisms remain unclear. In these contexts, our results in this paper may suggest that the transcription activator ATF/E4TF3 can recruit the CKII activity in the promoter region and then CKII can phosphorylate factors which affect the efficiency of elongation rather than the rate of initiation. In addition, our preliminary results indicated that action of CKII is really involved in transcription and the inhibition by DRB is due to the inhibition of the CKII activity (53). Therefore, we believe that analysis of an inhibition mechanism by DRB helps us to investigate the nature of CKII in transcription.

Recently, P-TEF (positive transcription elongation factor) was identified by reconstruction of DRB-sensitive transcription using *Drosophila* transcription factors (14). P-TEF was originally identified as a DRB-sensitive factor and its function was necessary for processivity (14). By fractionation of *Drosophila melanogaster* Kc cells, the P-TEF activity were separated into three activities; P-TEFa, factor 2 and P-TEFb and P-TEFb were purified (54). P-TEFb was composed of two subunits with apparent molecular masses of 124 and 43 kDa and essential for processivity *in vitro*. They showed that P-TEFb acts after initiation and is the limiting factor in the production of long run-off transcripts (54). Perhaps P-TEFb is a kinase since DRB is canonically a kinase inhibitor, however, the subunit composition of P-TEFb displays no obvious similarity to known kinases, especially CKII (54).

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