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Phosphorylation of ATF-1 enhances its DNA binding and transcription of the Na,K-ATPase α1 subunit gene promoter

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ABSTRACT

Transcriptional activity of both ATF-1 and CREB is enhanced by protein phosphorylation. While enhancement has been attributed to an increase in binding affinity for a co-activator (CBP), induction of the DNA binding activity by phosphorylation is an open question. Using the Na,K-ATPase α1 subunit gene promoter, which has an asymmetrical ATF/CRE site, we analyzed the effect of phosphorylation on DNA binding activity of the ATF-1–CREB heterodimer. Dephosphorylation of the heterodimer in nuclear extracts reduced binding to the ATF/CRE site. Phosphorylation of ATF-1 at Ser63 enhanced its binding to the ATF/CRE site in both the homodimeric and heterodimeric forms. Transcription of the Na,K-ATPase α1 subunit gene promoter was also stimulated by phosphorylated ATF-1 *in vitro*.

INTRODUCTION

Activating transcription factor 1 (ATF-1) and cAMP response element binding protein (CREB) are members of the ATF/CREB family, which belongs to the basic leucine zipper superfamily (reviewed in 1,2). They bind to the DNA sequence TGACG, known as the cAMP response element (CRE) within the promoters of many cAMP-inducible genes or as the activation transcriptional factor (ATF) site, which is a crucial element in the response to adenovirus E1A protein. The ATF/CRE site mediates the action of not only cAMP and E1A protein but also Ca^{2+} (3), transforming growth factor β (4) and type I human T cell leukemia virus Tax protein (5,6). It has been shown that the ATF/CRE site is also essential for basal transcriptional activity of many promoters (7–11).

ATF-1, CREB and CRE modulator (CREM) constitute a subgroup within the ATF/CREB family defined by their amino acid sequence similarity and their ability to heterodimerize with each other (reviewed in 1,2). Phosphorylation of both ATF-1 and CREB by cAMP-dependent protein kinase (PKA) or Ca^{2+}-calmodulin-dependent protein kinases (CaMK) I, II and IV enhances transcription in transient transfection assays (12–20) or in *in vitro* transcription assays (13). The molecular mechanism of this activation is assumed to be that phosphorylation of ATF-1 or CREB enhances their binding affinity for transcriptional co-activators. Recent results have suggested that only the phosphorylated forms of ATF-1 and CREB bind to the transcriptional co-activator CREB binding protein (CBP; 20–22). CBP was shown to interact with a basal transcriptional factor, TFIIB, through its C-terminal transactivation domain (22). It has been suggested that phosphorylation of ATF-1 and CREB does not affect DNA binding activity (23,24). However, Nichols *et al.* provided evidence that DNA binding by CREB, especially to the asymmetrical ATF/CRE site, was enhanced by phosphorylation (25). It is important, therefore, to elucidate whether phosphorylation of ATF-1 affects binding to the asymmetrical ATF/CRE site.

In this paper we examine the effect of ATF-1 and/or CREB phosphorylation on DNA binding and transcription using the Na,K-ATPase α1 subunit gene (*Atp1a1*) promoter as a model. The *Atp1a1* promoter has an asymmetrical ATF/CRE site that is critical for promoter activity (11,26,27). Binding of the ATF-1–CREB heterodimer to this site is required for transcription in *in vitro* transcription assays using nuclear extracts from various rat tissues (11). We show that phosphorylation of ATF-1 enhances binding to the ATF/CRE site in *Atp1a1* in both the homodimeric and heterodimeric forms. Furthermore, we provide evidence that phosphorylation of ATF-1 is required for transcription of the *Atp1a1* promoter.

MATERIALS AND METHODS

Nuclear extracts and recombinant proteins

Nuclear extracts from kidney, brain and liver were prepared from Sprague–Dawley rats as previously described (11,28). ATF-1 and ATF-1S63A expressed in *Escherichia coli* were prepared as described previously (20) or purchased from Santa Cruz Biotechnology. CREB was induced in plasmid His6CREB-11d-transformed *E.coli* strain BL21(DE3) and purified on a Ni–NTA column (Qiagen) as described in the manufacturer's instructions. His6CREB-11d was a kind gift from Dr Jun-ichi Fujisawa (29). The purity of these proteins was analyzed by SDS–PAGE.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed as previously described (11,30). DNA probes ARE-P (5′-CTAGAACGGTGACGTGCACGCGTCTAG; 11), ARE-Psym (5′-CTAGAACGGTGACGTCAACGCGTCTAG) and Sp1-P (5′-CGC-

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GTGGGCGGAGCCA) were prepared by annealing synthetic oligonucleotides. The ATF/CRE and Sp1 sites are underlined. For dephosphorylation analysis, 5 µg each of the nuclear extracts were incubated with or without 0.12 U bacterial alkaline phosphatase (NEB) at 30°C for 10 min before EMSA. For quantification, autoradiograms were scanned and analyzed using the Discovery Series (PDI).

In vitro transcription assays

In vitro transcription assays were carried out as previously described (11). ARE-P binding proteins were depleted from kidney nuclear extract using an ARE-P2 conjugated column prepared from CNBr–Sepharose 4B (Pharmacia Biotech) as described in the manufacturer's instructions. ARE-P2 (5′-GGATCCAACGGTGACGTGCACGCGTGGATCC) was prepared by annealing synthetic oligonucleotides. The only differences between ARE-P2 and ARE-P are the 5′- and 3′-termini (underlined, *Bam*HI site instead of *Bfa*I site). For quantification, autoradiograms were scanned and analyzed using the Discovery Series.

In vitro phosphorylation of ATF-1 and CREB

ATF-1 and CREB were phosphorylated *in vitro* as described previously (25) with slight modifications. Briefly, ATF-1 or CREB was incubated with or without 3.2 U PKA catalytic subunit at 30°C for 30 min and further reaction was stopped by the addition of 12 ng PKA-specific kinase inhibitor (PKI; Sigma). The PKA catalytic subunit was prepared as described previously (20) or purchased from Sigma. Phosphorylated or unphosphorylated proteins were mixed in various combinations and incubated at 30°C for 30 min for dimer formation before EMSA. For comparative analyses between binding to the symmetrical and asymmetrical ATF/CRE sites and for *in vitro* transcription assays, ATF-1 and CREB were phosphorylated after dimer formation. Four hundred nanograms of ATF-1 or CREB or a mixture of 200 ng each of ATF-1 and CREB were incubated at 30°C for 30 min and then phosphorylated by 6.4 U PKA catalytic subunit as described above. After addition of 24 ng PKI to stop further reaction, the proteins were analyzed by EMSA or *in vitro* transcription assay. Two-dimensional tryptic phosphopeptide mapping was performed as described previously (20).

RESULTS

Dephosphorylation of the ATF-1–CREB heterodimer in nuclear extracts reduces binding to the ATF/CRE site

We previously identified the *cis*-regulatory elements for constitutive expression of *Atp1a1* as an ATF/CRE site in a position between –70 and –66 of the promoter (Fig. 1A; 11). The common factor which binds to the ATF/CRE site in nuclear extracts from rat tissues (kidney, brain and liver) was identified as the ATF-1–CREB heterodimer by EMSA using specific antibodies (11). Phosphorylation of ATF-1 and CREB by multiple kinases is known to enhance transcriptional activity. To examine the role of ATF-1 and/or CREB phosphorylation on *Atp1a1* expression, we first analyzed the phosphorylation status of the ATF-1–CREB heterodimer in the nuclear extracts. Rat kidney, brain and liver nuclear extracts were treated with alkaline phosphatase and analyzed by EMSA using synthetic oligonucleotide ARE-P as a probe (from position –75 to –57 of *Atp1a1*), which includes the

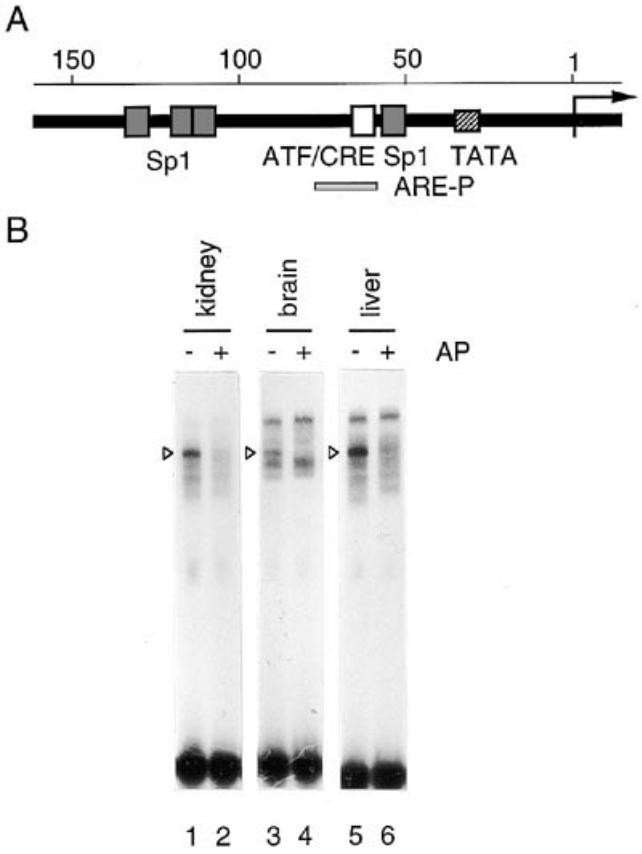

Figure 1. Phosphatase treatment of nuclear extracts reduced DNA binding of the ATF-1–CREB heterodimer. (**A**) The promoter elements of *Atp1a1*. The Sp1 sites, the ATF/CRE site and a TATA box-like sequence are indicated by shaded, open and striped boxes respectively. The region corresponding to the ARE-P probe is indicated by a shaded bar. (**B**) Effect of phosphatase treatment of nuclear extracts on DNA binding. Nuclear extracts from rat kidney (lanes 1 and 2), brain (lanes 3 and 4) and liver (lanes 5 and 6) were incubated without (lanes 1, 3 and 5) or with 0.12 U bacterial alkaline phosphatase (lanes 2, 4 and 6) at 30°C for 10 min. Then EMSA against ^{32}P-labeled ARE-P probes were performed using these extracts as described in Materials and Methods. Open arrowheads indicate complexes containing the ATF-1–CREB heterodimer.

ATF/CRE site (Fig. 1). Alkaline phosphatase treatment greatly reduced binding activity of the ATF-1–CREB heterodimer in all nuclear extracts (lanes 2, 4 and 6). The reduction was only seen in complexes formed by the ATF-1–CREB heterodimer (open arrowheads) and minor complexes in the brain and liver nuclear extracts were unaffected by phosphatase treatment. Thus, we conclude that the ATF-1–CREB heterodimer is phosphorylated in various rat tissues and that this phosphorylation is required for binding to the ATF/CRE site.

Phosphorylation of ATF-1 and CREB enhances binding to the ATF/CRE site

To elucidate which transcription factor in the ATF-1–CREB heterodimer must be phosphorylated for DNA binding, we performed EMSA using *in vitro* phosphorylated recombinant proteins. Recombinant ATF-1 and CREB were prepared from *E.coli* as described in Materials and Methods. Purity of each protein was >90%, as shown by Coomassie brilliant blue staining after SDS–PAGE (Fig. 2A). After *in vitro* phosphorylation using the PKA catalytic subunit, the proteins were mixed together to

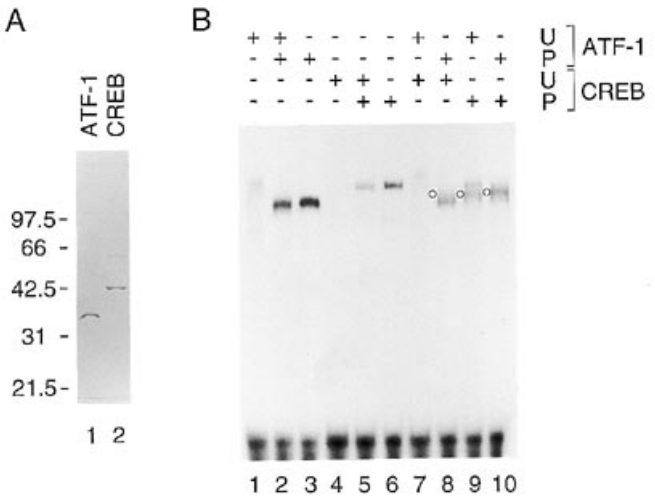

Figure 2. Phosphorylation of ATF-1 and/or CREB enhances DNA binding. (**A**) SDS–PAGE analysis of the *E.coli* expressed recombinant ATF-1 and CREB used in this paper. Four hundred nanograms each of the purified ATF-1 (lane 1) and CREB (lane 2) were analyzed by 10% SDS–PAGE. Positions of the molecular size markers are indicated in kilodaltons. (**B**) Effect of phosphorylation of ATF-1 and/or CREB on DNA binding. Two hundred nanograms each of the *in vitro* phosphorylated (P) and/or unphosphorylated (U) ATF-1 and CREB was mixed in combination as indicated (total 0.4 µg) and incubated at 30°C for 30 min. EMSA against ^{32}P-labeled ARE-P probes were performed using each mixture. The positions of complexes containing the ATF-1–CREB heterodimer are indicated by open circles.

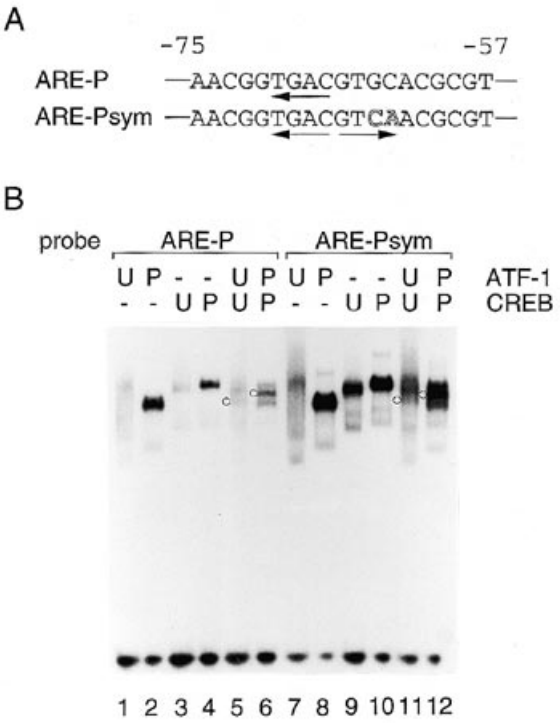

Figure 3. Phosphorylation of ATF-1 enhances binding to the symmetrical ATF/CRE site. (**A**) Nucleotide sequences of the ARE-P and ARE-Psym probes. –64G and –63C were replaced with C and A respectively (outlined characters) in the ARE-Psym probe. (**B**) Effect of phosphorylation of ATF-1 and CREB on binding to the symmetrical ATF/CRE site. After ATF-1 and CREB were mixed in combination as indicated (total 0.4 µg), each mixture was incubated with (P) or without (U) the PKA catalytic subunit. EMSA were performed against ^{32}P-labeled ARE-P (lanes 1–6) or ARE-Psym (lanes 7–12) probes using these mixtures. The positions of complexes containing the ATF-1–CREB heterodimer are indicated by open circles.

form either homodimer or heterodimer in various combinations. Further phosphorylation was inhibited by the addition of an excess amount of PKI before dimer formation. Unphosphorylated proteins were prepared by incubating without the PKA catalytic subunit under the same conditions as for phosphorylated proteins. No protein degradation was detected after the reaction as judged by SDS–PAGE analysis (data not shown). The results of EMSA using these dimers are shown in Figure 2B. Little DNA binding was observed for unphosphorylated ATF-1 or CREB in either the homodimeric or heterodimeric forms (lanes 1, 4 and 7), while phosphorylation of ATF-1 or CREB strongly enhanced DNA binding in both the homo- and heterodimeric forms (lanes 2,3, 5,6 and 8–10). Elevation of the ratio of phosphorylated to unphosphorylated proteins in the reaction mixture increased DNA binding in both the ATF-1 and CREB homodimers (lanes 3 and 6). A lower complex in lane 8 and an upper complex in lane 9 may be homodimers of phosphorylated ATF-1 and CREB respectively, as judged by their migration positions. Though the ATF-1–CREB heterodimer was formed efficiently from phosphorylated ATF-1 and CREB (lane 10), replacement of either one of them by unphosphorylated ATF-1 or CREB reduced DNA binding (lanes 8 and 9). The results demonstrate that phosphorylation of either ATF-1 or CREB enhances binding to the ATF/CRE site in both the homodimeric and heterodimeric forms and that phosphorylation of both ATF-1 and CREB is required for maximal DNA binding activity in the heterodimeric form.

Phosphorylation of ATF-1 enhances binding to both asymmetrical and symmetrical ATF-1/CRE sites

Nichols *et al.* classified the ATF/CRE site into two groups, symmetrical and asymmetrical (25). The symmetrical TGACGTCA ATF/CRE site is composed of two functional TGACG units that overlap on opposite DNA strands. The asymmetrical ATF/CRE site contains only one TGACG unit. The ATF/CRE site in the *Atp1a1* promoter has only one TGACG unit that forms an asymmetrical ATF/CRE site. Binding of CREB to the asymmetrical ATF/CRE site is more affected by phosphorylation than binding to the symmetrical ATF/CRE site (25). To examine the effect of ATF-1 phosphorylation on binding to the symmetrical ATF/CRE site, we substituted dinucleotide GC with CA in ARE-P to make the ATF/CRE site symmetrical (Fig. 3A, ARE-Psym). Using ARE-P and ARE-Psym as probes, we compared DNA binding activities of various ATF-1–CREB dimers by EMSA. To minimize the effect of phosphorylation on heterodimer formation as observed in Figure 2, we phosphorylated proteins after dimer formation. As shown in Figure 3B, binding of the ATF-1 homodimer to both ARE-P and ARE-Psym was greatly elevated by phosphorylation (10- and 16-fold increases respectively; lanes 1, 2 and 7, 8). DNA binding by the CREB homodimer was increased 4-fold for ARE-P (lanes 3 and 4), but only 1.6-fold for ARE-Psym (lanes 9 and 10) by phosphorylation. DNA binding of the ATF-1–CREB heterodimer was also enhanced by phosphorylation (3.4-fold increase for ARE-P and 5-fold increase for ARE-Psym; lanes 7, 8 and 11, 12, open circles). The major complexes in lanes 5 and 11 may be

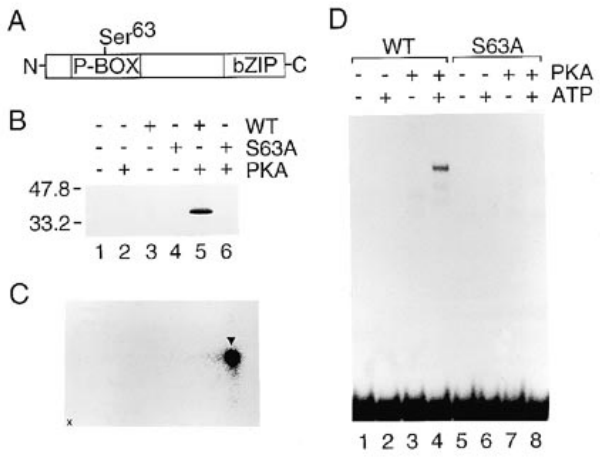

Figure 4. A point mutation at Ser63 in ATF-1 abolished enhancement of DNA binding by phosphorylation. (**A**) Schematic diagram of ATF-1. P-BOX, the phosphorylation box containing a PKA phosphorylation site at Ser63; bZIP, the basic region and the leucine zipper domain required for DNA binding and dimerization. (**B**) Phosphorylation analyses of wild-type ATF-1 (WT) and ATF-1S63A (S63A) by the PKA catalytic subunit. Five hundred nanograms each of wild-type ATF-1 and ATF-1S63A were incubated without (lanes 1, 3 and 4) or with (lanes 2, 5 and 6) the PKA catalytic subunit in the presence of [γ-^{32}P]ATP at 30°C for 30 min. ^{32}P-Labeled proteins were analyzed by 10% SDS–PAGE and visualized by autoradiography. Positions of the molecular size markers are indicated in kilodaltons. (**C**) Two-dimensional tryptic phosphopeptide mapping of ATF-1. Wild-type ATF-1 was phosphorylated *in vitro* by the PKA catalytic subunit in the presence of [γ-^{32}P]ATP. The phosphorylated proteins were purified by SDS–PAGE and digested with trypsin. ^{32}P-Labeled tryptic peptides were separated by high voltage electrophoresis in the first dimension (horizontal axis) and by thin layer chromatography (vertical axis) and then visualized by autoradiography. A closed arrowhead and × indicate the phosphopeptide including Ser63 and the origin of the two-dimensional separation respectively. (**D**) Effect of the point mutation at Ser63 in ATF-1 on binding to ARE-P. Twenty nanograms each of wild-type ATF-1 (WT) or ATF-1S63A (S63A) were incubated without (lanes 1, 2, 5 and 6) or with (lanes 3, 4, 7 and 8) the PKA catalytic subunit in the absence (lanes 1, 3, 5 and 7) or presence (lanes 2, 4, 6 and 8) of 1 mM ATP. EMSA against ^{32}P-labeled ARE-P probes were performed using these mixtures.

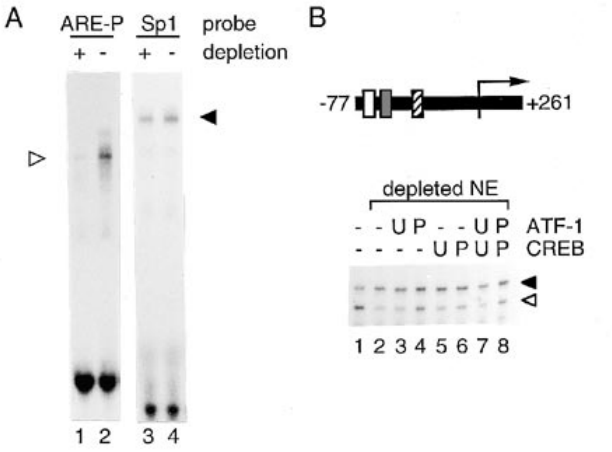

Figure 5. Phosphorylation of ATF-1 enhances transcription of the *Atp1a1* promoter. (**A**) Quantities of ARE-P binding activity in the nuclear extracts. EMSA were performed against either ^{32}P-labeled ARE-P (lanes 1 and 2) or Sp1-P (lanes 3 and 4) probes using 3 µg ARE-P binding protein-depleted (lanes 1 and 3) and undepleted (lanes 2 and 4) nuclear extracts from rat kidney. Sp1-P contains DNA sequences from position –51 to –36 of *Atp1a1*. Open and closed arrowheads indicate the positions of the shifted complexes with the ARE-P or Sp1 probes respectively. (**B**) Effect of phosphorylation of ATF-1 and CREB on transcription of the *Atp1a1* promoter. Four hundred nanograms of pA1U-77LF and 20 ng pAdMLCAT were transcribed using 30 µg each of the ARE-P binding protein-depleted (lanes 2–8) or undepleted (lane 1) nuclear extracts from rat kidney. Unphosphorylated (U) and/or phosphorylated (P) dimers composed of the indicated proteins (total 30 ng) were added to the nuclear extracts. The ATF/CRE site, the Sp1 site and a TATA box-like sequence are indicated by open, shaded and striped boxes respectively. The positions of specific transcripts from pA1U-77LF and pAdMLCAT are shown by open and closed arrowheads respectively.

unphosphorylated CREB homodimers, as judged by their migration positions. Binding to ARE-Psym was always stronger (between 3- and 13-fold) than that to ARE-P in all combinations. We conclude that phosphorylation of ATF-1 enhances binding to both asymmetrical and symmetrical ATF/CRE sites.

Phosphorylation at Ser63 in ATF-1 is essential for efficient DNA binding

A region of ~50 amino acids, named the phosphorylation box or P-BOX, in ATF-1 contains a number of serines that can be phosphorylated by various kinases (see Fig. 4A; 1,2). To elucidate the sites of phosphorylation by the PKA catalytic subunit in ATF-1, we phosphorylated ATF-1 with [γ-^{32}P]ATP, followed by tryptic phosphopeptide mapping. ^{32}P was incorporated into ATF-1 only in the presence of the PKA catalytic subunit (Fig. 4B, lane 5). As shown in Figure 4C, ^{32}P was exclusively incorporated into a single tryptic peptide that was identified as a nonadecapeptide from Gly56 to Glu74 (GILARRPSYRKILKDLSSE) in the P-BOX (20). This nonadecapeptide contains Ser63, which was assumed to be the phosphorylation site for PKA. The amino acid sequence around Ser63 in ATF-1 is highly homologous to that around Ser133 in CREB (1,2). Ser133 in CREB is known to be the phosphorylation target site for PKA required for the activation of gene transcription by CREB (12,31). To examine whether the PKA catalytic subunit phosphorylates ATF-1 at Ser63, we used ATF-1S63A, in which Ser63 was substituted by alanine, in *in vitro* phosphorylation analyses. As shown in Figure 4B, wild-type ATF-1 (lane 5) but not ATF-1S63A (lane 6) was phosphorylated by the PKA catalytic subunit. These results indicate that Ser63 is the only site to be phosphorylated by the PKA catalytic subunit *in vitro*. We next compared the effect of phosphorylation reactions between wild-type ATF-1 and ATF-1S63A on binding to the ATF/CRE site. Figure 4D showed that phosphorylation by the PKA catalytic subunit enhanced the DNA binding activity of wild-type ATF-1 but not that of ATF-1S63A. The enhancement was dependent on the presence of both kinase and ATP (lanes 1–4). These results indicate that the PKA catalytic subunit phosphorylates ATF-1 at Ser63 and that phosphorylation is essential for efficient DNA binding by ATF-1.

Phosphorylation of ATF-1 activates transcription of the *Atp1a1* promoter *in vitro*

To elucidate the effect of ATF-1 phosphorylation on transcription, we next performed an *in vitro* transcription assay using the *Atp1a1* promoter as template. The *Atp1a1* promoter was efficiently transcribed *in vitro* using nuclear extracts from rat kidney, brain and liver (11). Transcription was dependent on the ATF/CRE site of the promoter and on the ATF-1–CREB heterodimer in nuclear

extracts. We chose nuclear extract from kidney for the analysis, because the ATF-1–CREB heterodimer is the only ATF/CRE site binding complex in this extract as judged by EMSA (see Fig. 1B; 11). The ARE-P binding proteins were depleted from kidney nuclear extract using a DNA affinity column as described in Materials and Methods. The resulting nuclear extract had little ARE-P binding activity, as shown in Figure 5A (14% of the undepleted extract; lanes 1 and 2). The depletion was ARE-P specific, since Sp1 site binding activity was unaffected (78% of the undepleted extract; lanes 3 and 4). We used this depleted nuclear extract for *in vitro* transcription assays (Fig. 5B). Plasmid pA1U-77LF (11), which has nt –77 to +261 of *Atp1a1*, was used as template. The adenovirus major late (AdML) promoter was used as a control template (11,32). Transcription from pA1U-77LF was reduced to 28% by depletion of the ARE-P binding proteins (lanes 1 and 2, open arrowhead). This reduction was comparable with that of our previous observation in transcription competition assays using ARE-P (11). The addition of unphosphorylated ATF-1 homodimer, CREB homodimer or ATF-1– CREB heterodimer had little effect on transcription (1.2-, 1- and 0.9-fold increase respectively; lanes 3, 5 and 7), while addition of the phosphorylated ATF-1 homodimer and ATF-1–CREB heterodimer stimulated transcription (2.3- and 1.9-fold increase respectively; lanes 4 and 8). Phosphorylated CREB homodimer showed intermediate stimulation of transcription (1.5-fold increase; lane 6). We conclude that phosphorylation of ATF-1 is indispensable for transcription of the *Atp1a1* promoter.

DISCUSSION

In this paper we have demonstrated that phosphorylation of ATF-1 stimulates DNA binding by the following criteria. (i) Dephosphorylation of nuclear extracts reduced DNA binding by endogenous ATF-1–CREB heterodimers (Fig. 1B). Previous observation by Merino *et al.* also suggested that DNA binding by ATF-1 in HeLa cell nuclear extracts is reduced by dephosphorylation (33). (ii) EMSA using *E.coli* expressed recombinant proteins showed that phosphorylation of ATF-1 greatly enhanced binding to the ATF/CRE site in both the homodimeric and heterodimeric forms (Fig. 2B). (iii) A point mutation at Ser63 in ATF-1, the only site for *in vitro* phosphorylation by the PKA catalytic subunit, abolished the enhancement of DNA binding by phosphorylation (Fig. 4D). This conclusion is inconsistent with a previous report by Hurst *et al.* (24), in which they reported that dephosphorylation of ATF-1 in HeLa cell nuclear extracts did not reduce DNA binding activity by Southwestern analysis and by binding analysis to ATF/CRE site affinity beads. However, it was not demonstrated that ATF-1 had been renatured quantitatively to form homodimer on the blotted filter in the Southwestern analysis. Moreover, binding of ATF-1 to the ATF/CRE site was apparently reduced in their binding analysis to DNA affinity beads.

It is possible that phosphorylation of both ATF-1 and CREB potentiates heterodimer formation leading to enhancement of DNA binding. Indeed, we observed that homodimer consisting of phosphorylated ATF-1 or CREB was efficiently formed in the presence of an unphosphorylated partner for heterodimerization, while the heterodimer was preferentially formed in the presence of a phosphorylated partner (Fig. 2B, lanes 8–10). Dimer formation has been shown to be required for enhancement of the transcriptional activity of ATF-1 by PKA (16). Deletion of the leucine zipper domain, a dimerization domain, of Gal4-fused ATF-1 resulted in no enhancement in response to PKA (16). This observation can be explained if we consider that enhancement of DNA binding by ATF-1 phosphorylation is mediated by elevation of dimer formation. The effects of phosphorylation on dimer formation remain to be elucidated.

Though phosphorylation of CREB at Ser133 enhanced its binding to the asymmetrical site, binding to the symmetrical ATF/CRE site was hardly affected by phosphorylation, consistent with previous reports (25,34). In contrast to CREB, phosphorylation of ATF-1 at Ser63 dramatically enhanced binding to both the asymmetrical and symmetrical ATF/CRE sites to the same extent (Fig. 3B). These observations suggest that phosphorylation of ATF-1 may be required for binding to any kind of ATF/CRE site, which is not the case for CREB.

It has been considered that the N-terminal region (NTR) of ATF-1, which has no similarity with CREB, plays a significant role in phosphorylation-induced activation of ATF-1 (35). The NTR was suggested to be important for a phosphorylation-induced conformational change (35) and deletion of this region stabilized DNA binding (16). We used an NTR-deleted ATF-1 in this study, except for the experiments shown in Figure 4. Nevertheless, activation of ATF-1 by phosphorylation was observed for both DNA binding and transcription. DNA binding by full-length ATF-1 was also enhanced by phosphorylation (Fig. 4D). This enhancement was comparable with that in the case of NTR-deleted ATF-1 (data not shown). Our results suggest that the NTR is not essential for phosphorylation-induced activation of ATF-1.

Ser63 in ATF-1 is the only site that can be phosphorylated by the PKA catalytic subunit *in vitro* (Fig. 4B and C) and phosphorylation at Ser63 is essential for efficient binding to the ATF/CRE site (Fig. 4D). As reported previously, CaMK II also phosphorylates ATF-1 *in vitro* exclusively at Ser63 and activates ATF-1-dependent transcription in transient transfection assays (20). It is possible that elevation of the transcriptional activity of ATF-1 by CaMK II is mediated partially by an enhancement of DNA binding.

Several groups have demonstrated that unphosphorylated ATF-1 is unable to activate transcription from the somatostatin promoter, containing a symmetrical ATF/CRE site, by transient transfection experiments (15,17,20). Transcriptional activity of ATF-1 was induced only when it was co-expressed with activated forms of PKA or CaMK II in cells. This is consistent with our results in *in vitro* transcription assays that only the phosphorylated and not the unphosphorylated form of ATF-1 homodimer or ATF-1–CREB heterodimer had the ability to activate transcription (Fig. 5B).

Previously we reported that the ATF-1–CREB heterodimer is involved in the constitutive expression of *Atp1a1* (11). Transcription of the *Atp1a1* promoter is regulated by intracellular cAMP (36,37) or Ca^{2+} (38) levels. We speculate that this induction is mediated by phosphorylation of ATF-1 and CREB in the ATF-1–CREB heterodimer by PKA or CaMK. The role of the ATF-1–CREB heterodimer in cAMP- or Ca^{2+}-mediated induction of *Atp1a1* expression remains to be elucidated.

Transcriptional activation by phosphorylated CREB homodimer was weak in our experiments (Fig. 5B, lane 6). Wadzinski *et al.* reported that protein phosphatase activity in nuclear extract from rat liver was strong enough to dephosphorylate CREB during *in vitro* transcription assays (39). It is also possible that phosphorylated CREB was unstable in the nuclear extract from rat kidney,

while phosphorylated ATF-1 was stable in the extract. We prefer this possibility because constitutive expression of *Atp1a1* can be explained by the stability of phosphorylated ATF-1 in various tissues.

Here we provide direct evidence that phosphorylated ATF-1–CREB heterodimer is indispensable for transcription mediated by the ATF/CRE site in the *Atp1a1* promoter. Since the *Atp1a1* promoter is constitutively active in various cells, we originally hypothesized that phosphorylation of the ATF-1–CREB heterodimer would have little effect on transcription. However, our results indicate that ATF-1 phosphorylation is, in fact, essential for both DNA binding and transcription.

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