Identification of mouse Jun dimerization protein 2 as a novel repressor of ATF-2[1]

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Abstract A mouse cDNA that encodes a DNA-binding protein was identified by yeast two-hybrid screening, using activating transcription factor-2 (ATF-2) as the bait. The protein contained a bZIP (basic amino acid-leucine zipper region) domain and its amino acid sequence was almost identical to that of rat Jun dimerization protein 2 (JDP2). Mouse JDP2 interacted with ATF-2 both in vitro and in vivo via its bZIP domain. It was encoded by a single gene and various transcripts were expressed in all tested tissues of adult mice, as well as in embryos, albeit at different levels in various tissues. Furthermore, mouse JDP2 bound to the cAMP-response element (CRE) as a homodimer or as a heterodimer with ATF-2, and repressed CRE-dependent transcription that was mediated by ATF-2. JDP2 was identified as a novel repressor protein that affects ATF-2-mediated transcription. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Activating transcription factor-2; Transcription factor; DNA-binding; cAMP-response element-dependent transcription

1. Introduction

Activating transcription factor-2 (ATF-2) is a member of the ATF/CREB family of transcription factors. It contains a basic amino acid-leucine zipper motif (bZIP) and mediates a variety of transcriptional responses that are associated with the proliferation and differentiation of cells, tumorigenesis and apoptosis [1–6]. ATF-2 forms homodimers and heterodimers with other members of the ATF family, as well as with members of the Jun/Fos family, through the leucine zipper motif [7–14]. Furthermore, it binds to the cAMP-response element (CRE) and to CRE-like elements [3–5].

It has been proposed that each homo- or heterodimer that includes ATF-2 is functionally unique in terms of the capacity to regulate transcription and ability to target a particular subset of CRE/ATF- or Ap-1-regulated genes [11]. ATF-2 has been implicated as a mediator in the transcriptional response to the adenovirus E1A protein [3–5], the Rb protein [12] and genotoxic stress [13]. However, the biological role of ATF-2 is poorly understood. ATF-2 knockout mice exhibit reduced postnatal viability and growth, with a defect in endochondral ossification and ataxia, that is accompanied by decreases in the number of cerebellar Purkinje cells [14], and symptoms of severe respiratory distress, which resemble those of severe human meconium aspiration syndrome [15].

The transactivation capacity of ATF-2 is also regulated by phosphorylation, which is catalyzed, in part, by the Jun amino-terminal kinase/stress-activated protein kinase (JNK/SAPK) and by mitogen-activated protein kinases in the p38 family, in response to cellular stress [13,16–18]. SAPKs, such as JNKs and p38, phosphorylate ATF-2 at residues Thr-69, Thr-71 and Ser-90, which are located close to the amino-terminal transcriptional activation domain. This phosphorylation stimulates the transactivating capacity of ATF-2 [18]. The conserved region 3 (CR3) of adenovirus E1A protein interacts with the leucine zipper of ATF-2, putatively inducing a conformational change that results in exposure of the amino-terminal activation domain [19]. This type of activation involves the phosphorylation of threonine or serine residues in the amino-terminal region of ATF-2. The E1A protein can also activate transcription of the *c-jun* gene in a CR1-dependent manner. This activation is mediated by two Ap-1/ATF-like elements in promoters, namely, jun1-TRE (TGACATCA) and jun2-TRE (TTACCTCA), which preferentially bind heterodimers composed of pre-existing c-Jun and ATF-2 or an ATF-2-related factor [13,20]. Activation that is dependent on CR1 does not depend strictly on the presence of Thr-69 and Thr-71 at the amino-terminus of ATF-2 [19]. The mechanism of the CR1-dependent activation of the *c-jun* gene, that is mediated by ATF-2, is still unclear.

We have shown that ATF-2 and p300 cooperate in retinoic acid (RA)-mediated and in E1A-mediated transcription of the *c-jun* gene during the differentiation of mouse embryonal carcinoma F9 cells [21–23]. We also found that phosphorylation of p300 and of ATF-2 is associated with the RA- and the E1A-mediated differentiation of F9 cells. We identified two

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complexes, designated differentiation regulatory factor-1 (DRF1) and DRF2, that bound specifically to a differentiation-response element, which was both necessary and sufficient for the RA- and the E1A-mediated enhancement of the expression of the *c-jun* gene [24]. However, the level of transactivation of the *c-jun* promoter by p300 and ATF-2 was consistently lower than the level achieved during RA- and during E1A-dependent activation of the *c-jun* gene. UV cross-linking studies also indicated the presence of other proteins in the DRF complexes [21]. Therefore, in this study, in an attempt to identify other proteins in the DRF complexes during the differentiation of F9 cells, we used a yeast two-hybrid assay with ATF-2 as the bait. We describe here the identification of mouse Jun dimerization protein 2 (JDP2) as a direct partner of ATF-2, that represses the CRE-dependent transcription mediated by ATF-2.

2. Materials and methods

2.1. Plasmids

pGBT9-ATF-2, which included the full-length cDNA for ATF-2 fused to the GAL4 DNA-binding domain (GAL4DBD), was prepared as described elsewhere [25]. pGEX-ATF-2 and pECE-ATF-2 were gifts from M. Green (University of Massachusetts Medical Center, Worcester, MA, USA). pGEX-JDP2 was generated by ligating the PCR-amplified fragment of DNA that included the coding sequence (amino acids 1–163) of JDP2 into pGEX-4T-1 (Amersham Pharmacia Biotech., Buckinghamshire, UK), in frame. CRE/tk-CAT was provided by M.R. Montimiy (Salk Institute, La Jolla, CA, USA) [26]. mCRE/tk-CAT was constructed using site-directed mutagenesis to change the CRE site (5′-TGACGTCA-3′) into an mCRE site (5′-TGCAGTCA-3′). pcDNA4-JDP2 was prepared by subcloning the JDP2-coding region, which had been amplified by PCR, into pcDNA4/HisMax (Invitrogen Co., Carlsbad, CA, USA). pBluescript (pBS)-ATF-2bZIP and pGEX-JDP2bZIP were generated by inserting the bZIP DNA fragment of ATF-2 (amino acids 350–415) or of JDP2 (amino acids 70–135), respectively, into the appropriate site of the pBS and the pGEX vector. pGEX-JDP2bZIP(L34V) was also constructed by site-directed mutagenesis in which the third and the fourth leucine in the bZIP region of JDP2 were converted to valine.

2.2. Yeast two-hybrid screening

Yeast two-hybrid screening was carried out according to the manufacturer's instructions (Clontech Inc., Palo Alto, CA, USA). Yeast strain HF7C, which included both *HIS3* and *LacZ* reporter genes under the control of the GAL4 upstream activating sequence (UAS), was cotransformed with the bait plasmid pGBT9-ATF-2 and with a mouse 11-day embryo MATCHMAKER™ cDNA library constructed in pACT2 (Clontech Inc.) with inserts fused to the GAL4 activation domain (GAL4AD). Transformants were grown in selective medium without tryptophan (Trp; pGBT9-specific selective marker), leucine (Leu; pACT2-specific selective marker) and histidine (His; specific for the reporter) at 30°C. Colonies that both expressed the *HIS3* reporter as a result of interaction of two-hybrid proteins, and grew in this selection medium were further analyzed for β-galactosidase activity due to the *LacZ* derived from the corresponding reporter. The plasmids from His+/β-galactosidase+ colonies were then used to transform *Escherichia coli* HB101 by electroporation. The transformants were grown on M9 (−Leu) media for selection of colonies that contained pACT2. Then the plasmids were purified and cDNA inserts were sequenced. To define the unknown sequences at the 5′-ends of the cDNAs from clones isolated by the two-hybrid screening assay, we performed 5′-rapid amplification of cDNA ends (RACE) using a Marathon™ cDNA amplification kit (Clonetech Inc.) in accordance with the manufacturer's protocol. Poly(A)+ RNA was purified from F9 cells with a 'Quick prep. micro purification' kit (Amersham Pharmacia Biotech.). The sequence of the specific primer for 5′-RACE was 5′-AGTTTGTCACGTGCCTGGGAGGGGC-3′.

2.3. Translation in vitro and examination of the binding of ATF-2 and JDP2

In vitro transcription/translation of pBS-ATF-2, pBS-JDP2 or pBS-ATF-2bZIP was performed with a kit from Promega (Madison, WI, USA) according to the manufacturer's protocol. Plasmids were transcribed with T3/T7 RNA polymerase and proteins were translated in reticulocyte lysates in the presence of [³⁵S]methionine. The efficiency of translation was monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were quantitated with a Bio-Imaging Analyzer (BAS 2000; Fuji, Tokyo, Japan). For protein-binding assays, 2 μg of GST fusion protein, that included JDP2, ATF-2, JDP2bZIP or JDP2bZIP(L34V), was mixed with 5 μl of reticulocyte lysate, together with glutathione-Sepharose beads (Amersham Pharmacia Biotech.), for 2 h at 4°C. The beads were then washed extensively and protein complexes were eluted with buffer containing 10 mM glutathione for analysis by SDS–PAGE. Gels were dried prior to exposure to X-ray film.

2.4. Binding of ATF-2 and JDP2 in vivo

Nuclear extracts of F9 cells, which had been transfected with the plasmid for ATF-2 together with either the plasmid for histidine-tagged JDP2 or the empty vector alone using FuGENE6™ transfection reagent (Boehringer Mannheim, Mannheim, Germany), were incubated with TALON metal affinity resins (Clonetech Inc.) for 30 min at 4°C. The resins were then washed extensively and the bound proteins were eluted with the buffer containing 50 mM imidazole for 20 min at room temperature. The samples were resolved on 10% SDS gels and subjected to Western blot analysis with anti-ATF-2 antibody (Santa Cruz Biotech. Inc., Santa Cruz, CA, USA).

2.5. Northern blotting analysis

A mouse multiple-tissue Northern (MTN) blot prepared with poly-(A)+ RNA from eight different mouse tissues and mouse embryonic MTN blot prepared with poly(A)+ RNA from different embryo tissues were obtained from Clonetech Inc. and hybridizations were performed according to the manufacturer's instructions. The probe was radiolabeled with [α-³²P]dCTP by the random-priming method of Feinberg and Vogelstein [27,28].

2.6. Culture and transfection of cells and assay of CAT activity

NIH3T3 cells were grown in Dulbecco's modified Eagle's medium that had been supplemented with 10% fetal bovine serum (Gibco-BRL, Rockville, MD, USA). Cells were transfected with plasmid DNA using the FuGENE6™ transfection reagent according to the manufacturer's protocol. Cells were harvested 24 h after transfection and assays of CAT activity were performed as described elsewhere [24]. The extent of conversion of chloramphenicol to its acetylated forms was determined with the Bio-Imaging analyzer. The ratio of CAT activity to that of β-galactosidase was used for normalization of results.

2.7. Electrophoretic mobility shift assay (EMSA)

Plasmids pGEX-ATF-2, pGEX-JDP2 and pGEX were used to generate GST-ATF-2, GST-JDP2 and glutathione S-transferase (GST) and the proteins were purified as described elsewhere [21]. The proteins in various combinations were incubated in binding buffer (20 mM Tris (pH 7.5), 2 mM MgCl₂, 0.5 mM EDTA, 10% glycerol, 0.5 mM dithiothreitol, 25 mM NaCl, 0.5 μg poly(dI-dC)) for 5 min at room temperature, and a ³²P-radiolabeled double-stranded CRE oligodeoxynucleotide probe was added to the reaction mixture. After a 20-min incubation at room temperature, each sample was resolved on a 5% non-denaturing polyacrylamide gel in 0.5×TBE buffer, with subsequent autoradiography as described elsewhere [24]. Competition experiments were performed by prior incubation of the various proteins with a non-radiolabeled competitor oligodeoxynucleotide for 20 min. The sequence of the DNA probe was 5′-AGCTCCCGT-GACGTCACCCG-3′, corresponding to the CRE that extends from nucleotide (nt) −177 to nt −162 in the promoter of the gene for fibronectin from mouse [29]. To examine the formation of a JDP2 heterodimer with ATF-2, the EMSA using in vitro translated ATF-2 and JDP2 was performed as described elsewhere [32].

2.8. Determination of the nucleotide sequence of JDP2 cDNA

The nucleotide sequence of both strands of JDP2 cDNA was determined with a BigDye™ Terminator Cycle Sequencing kit (PE-Applied Biosystems Inc., Foster City, CA, USA). Nucleotide and deduced amino acid sequences were analyzed by a BLAST search [30,31] of the SwissProt and GenBank databases.
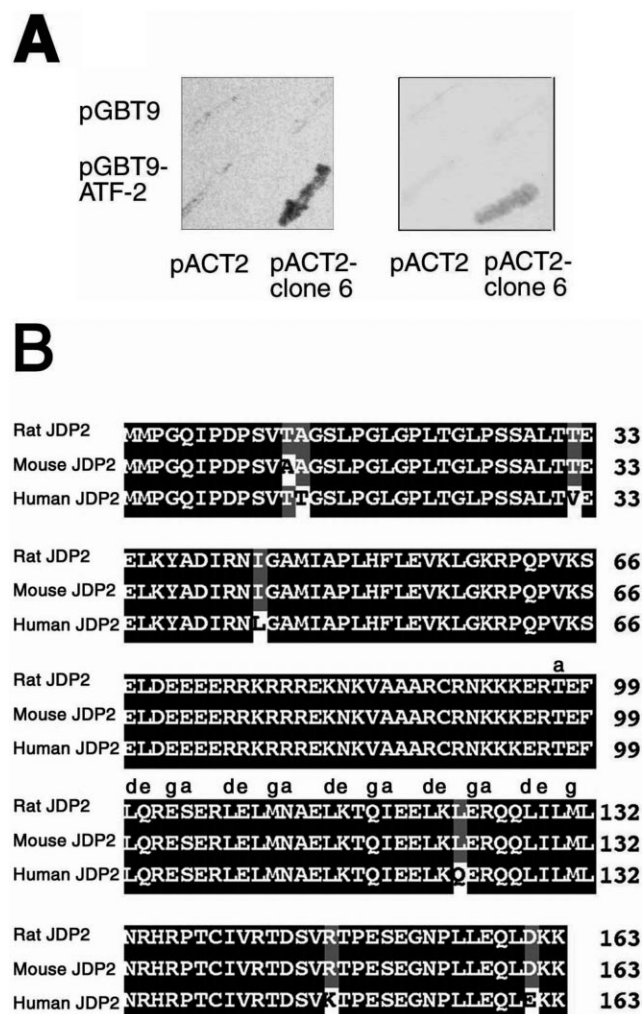
Fig. 1. Isolation of ATF-2-interacting proteins. (A) Isolation of mouse JDP2 as the partner of ATF-2. The GAL4AD-tagged mouse 11-day embryo cDNA library was used to cotransform HF7C yeast cells together with pGBT9-ATF-2, in which the cDNA for the DNA-binding domain (DBD) of GAL4 was fused to ATF-2 cDNA. Yeast HF7C cells contained *HIS3* and *LacZ* reporter genes under the control of the UAS of GAL4. Left panel: transformants coexpressing the pACT2-clone 6 and pGBT9-ATF-2 that grew on medium lacking histidine, as a result of expression of the *HIS3* reporter via interaction of ATF-2 and the protein encoded by clone 6. Negative controls are also shown. Right panel: β-galactosidase activity in HF7C yeast cells that expressed GAL4AD (pACT2) or the GAL4AD-clone 6 (pACT2-clone 6) fusion protein in the presence of GAL4DBD (pGBT9) or GAL4DBD-ATF-2 (pGBT9-ATF-2). (B) Comparison of the amino acid sequences among various JDP2 proteins. Identical sequences of three JDP2 proteins are shown by black boxes and two of them were indicated by gray boxes. The sequence of human JDP2 was deduced from the sequences of two genomic DNAs. a, d, e and g correspond to the positions of amino acid residues in α-helix of bZIP region, as previously demonstrated in the case of c-Jun and c-Fos [33,34].

3. Results

3.1. Isolation of proteins that interact with ATF-2

To isolate proteins that interacted with the ATF-2, we screened a mouse 11-day embryo cDNA library using the yeast two-hybrid system with cDNA for ATF-2 as the bait. We screened approximately 1×10^6 yeast transformants and identified 150 colonies by growth on selection medium that

lacked histidine. Sixteen of these clones were scored as positive for β-galactosidase activity and we determined the nucleotide sequences of these clones. Among the clones identified, clone 6 was selected for further characterization. As shown in Fig. 1A, the yeast transformant that coexpressed pACT2-clone 6 and pGBT9-ATF-2 grew on medium that lacked histidine (left panel). Activation of the *LacZ* reporter gene was determined by measuring β-galactosidase activity (right panel). When expressed alone, neither pGBT9-ATF-2 nor pACT2-clone 6 transactivated the *LacZ* reporter gene. By contrast, coexpression of the two plasmids resulted in strong expression of the reporter gene, indicating that the product of clone 6 and ATF-2 had interacted directly or indirectly in the yeast cells. Subsequent 5′-RACE led to the complete identification of the 5′-end sequences that encompassed the initiation codon and 137 bp of the 5′-untranslated region. The complete cDNA sequence of clone 6 (GenBank accession number AB034697) included 1.5-kb nucleotides and contained an open reading frame of 498 bp that corresponded to a polypeptide of 163 amino acids with a basic leucine zipper (bZIP) motif at its carboxyl-terminus.

A BLAST search revealed that the polypeptide that was encoded by clone 6 exhibited 99% identity with rat JDP2, which was initially isolated as a *c-jun* repressor by Aronheim et al. [32], and 96% alignment with human JDP2 deduced from a human genomic DNA sequence (GenBank accession number: AC009363 and AF111167; Fig. 1B). These observations suggest that clone 6 encodes the mouse homolog of JDP2. Sequence analysis also showed that no changes were presented in the critical position a/d and e/g in the α-helix of the bZIP domain that controls dimerization specificity (Fig. 1B) [33,34]. Furthermore, the comparison of the amino acid sequence revealed that JDP2 was approximately 53% homologous to ATF3, a transcriptional repressor, and its bZIP region was 75% homologous to that of ATF3 [35].

3.2. Binding of JDP2 to ATF-2 in vitro and in vivo

To confirm the interaction between the putative polypeptide JDP2 and ATF-2, we performed a binding study to explore the requirements for formation of a heterodimer between JDP2 and ATF-2. Three GST fusion proteins were synthesized and immobilized on glutathione-Sepharose beads. The beads were then incubated with reticulocyte lysates that contained [35S]methionine radiolabeled ATF-2 or JDP2 and, after extensive washing, the bound proteins were fractionated by SDS–PAGE. Fig. 2A shows that JDP2 bound to GST-ATF-2 beads and vice versa. However, JDP2 did not bind to the GST beads. We next examined the interaction between ATF-2 and JDP2 in vivo (Fig. 2B). Two plasmids for ATF-2, or for histidine-tagged JDP2, were cotransfected into F9 cells and histidine-tagged JDP2 protein was purified with TALON beads. Then the eluted proteins were analyzed by Western blotting with antibodies specific for ATF-2. ATF-2 was copurified with histidine-tagged JDP2 (lane 2), but not with histidine alone as a control (lane 1). A similar complementary experiment was performed and confirmed the results described above (data not shown). These results suggested that JDP2 interacted specifically with ATF-2 both in vitro and in vivo.

To determine further the interaction domain between JDP2 and ATF-2, we used mutant JDP2 and ATF-2 for the binding assay (Fig. 2C). Whereas the truncation mutants containing the bZIP domain, ATF-2bZIP and JDP2bZIP, bound

each other as efficiently as the wild-type, the mutant JDP2bZIP(L34V), in which the third and the fourth leucine in the bZIP region were changed to valine, failed to interact with ATF-2bZIP. These results imply that JDP2 and ATF-2 interact via their own bZIP domain.

3.3. Expression of JDP2 transcripts

We examined the tissue distribution of JDP2 transcripts using a mouse MTN blot (Fig. 3A). Transcripts were identified in all tissues examined, albeit at different levels. Interestingly, we detected at least 10 mRNAs, with lengths of 7.0 kb, 4.4 kb, 4.0 kb, 3.0 kb, 2.6 kb, 2.4 kb, 2.0 kb, 1.5 kb, 1.3 kb and 1.0 kb, respectively. The levels of the mRNAs in heart, brain, liver, kidney and testis were higher than in other tissues. The levels of JDP2 mRNA in spleen, lung and skeletal muscle were very low and the bands were only revealed after extended exposure (Fig. 3B). The 3.0-kb and 2.4-kb transcripts were most abundant in heart and brain tissue, respectively. In the liver and kidney, the 4.4-kb transcript was predominant, but the 3.0-kb and 2.4-kb mRNAs were also present at high levels. However, in testis, transcripts of 7.0 kb, 4.0 kb, 2.6 kb, 2.0 kb and 1.0 kb were detected as the major transcripts. Longer exposure revealed that transcripts of 3.0 kb, 2.0 kb and 1.5 kb were the major species in spleen, while transcripts of 4.4 kb, 3.0 kb and 2.0 kb were detected in the lung. The 7.0-kb, 2.6-kb and 1.3-kb transcripts were also detected in skeletal muscle. We also examined the pattern of expression of JDP2 in mouse embryos using mouse embryo MTN blots (Fig. 3C), and we detected mRNAs of 7.0 kb, 3.0 kb and 1.5 kb. Only the transcript of 3.0 kb was expressed in 7-day embryos and the level was very low, whereas transcripts of 1.5 kb and 3.0 kb were found in 11-day and 15-day embryos, respectively. All three transcripts were detected in 17-day embryos.

3.4. Repression by JDP2 of the CRE-dependent transcription that is mediated by ATF-2

To investigate the role of mouse JDP2 in the regulation of the transcriptional activity of ATF-2, we cotransfected NIH3T3 cells with various combinations of a CRE/tk-CAT

reporter construct, a plasmid that encoded JDP2 and a plasmid that encoded ATF-2, and monitored the CRE-mediated reporter activity in the presence and in the absence of JDP2 and ATF-2. As expected, ATF-2 by itself activated the transcription of the CRE/tk-CAT reporter gene (Fig. 4A). However, expression of JDP2 suppressed the ability of ATF-2 to activate the CRE/tk-CAT reporter gene (Fig. 4A). In a control experiment, we used the mCRE/tk-CAT construct, in
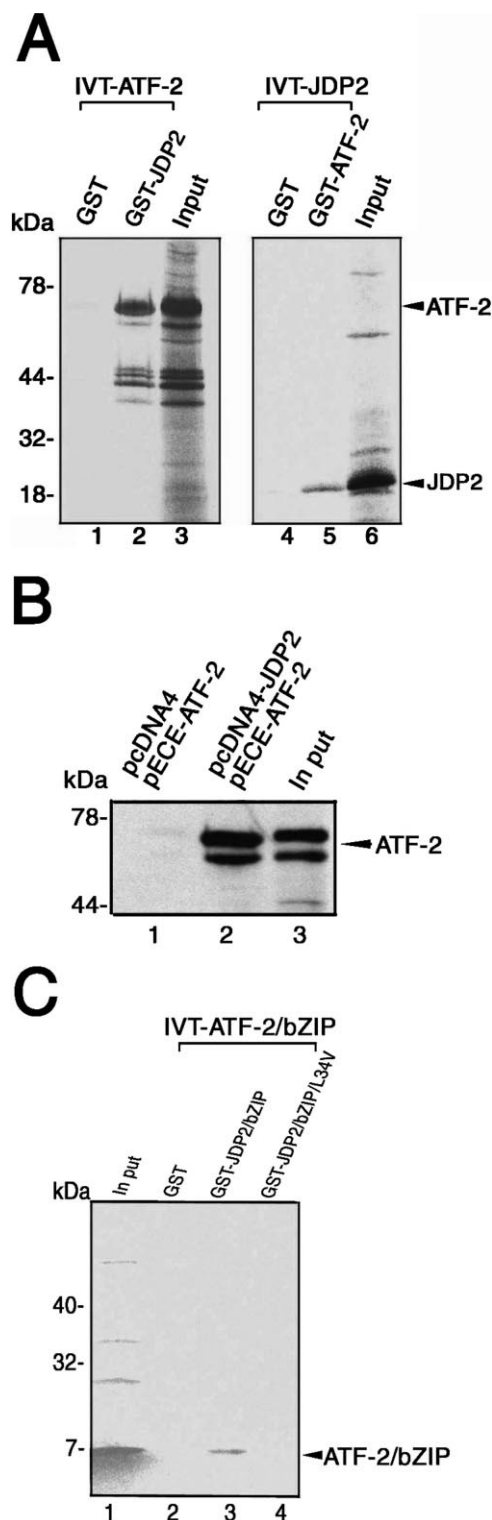

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Fig. 2. Interaction between ATF-2 and JDP2. (A) Selective interactions between ATF-2 and JDP2 in vitro. In vitro translated [35S]methionine-labeled ATF-2 (IVT-ATF-2) was incubated with GST-JDP2 (GST-JDP2) or GST (left panel) and vice versa (right panel). The amount of sample loaded in each input lane was equal to approximately 20% of the in vitro translated protein used for the binding assay. Bound ATF-2 and JDP2 are indicated by arrowheads and the mobilities and molecular masses of marker proteins are also shown. (B) Binding of JDP2 to ATF-2 in vivo. Whole cell lysates were prepared from F9 cells that had been cotransfected with an ATF-2 expression vector (pECE-ATF-2) and the plasmid (pcDNA4-JDP2) to express histidine-tagged JDP2 (lane 2), or empty vector (lane 1), and the histidine-tagged JDP2 were separated with TALON resins. The co-purified proteins were analyzed by Western blotting with antibodies specific for ATF-2. In lane 3, an aliquot of the cell lysates (approximately 5%) was directly used for Western blotting. (C) Interaction domain of ATF-2 and JDP2. In vitro translated [35S]methionine-labeled ATF-2/bZIP (IVT-ATF-2/bZIP) containing the bZIP domain of ATF-2 was incubated with GST-JDP2/bZIP containing the bZIP region of JDP2 (lane 3), GST-JDP2/bZIP(L34V), in which the third and the fourth leucine in the bZIP domain were changed to valine (lane 4), or GST (lane 2). Bound ATF-2/bZIP is indicated by an arrowhead and the molecular masses of marker proteins are also shown.
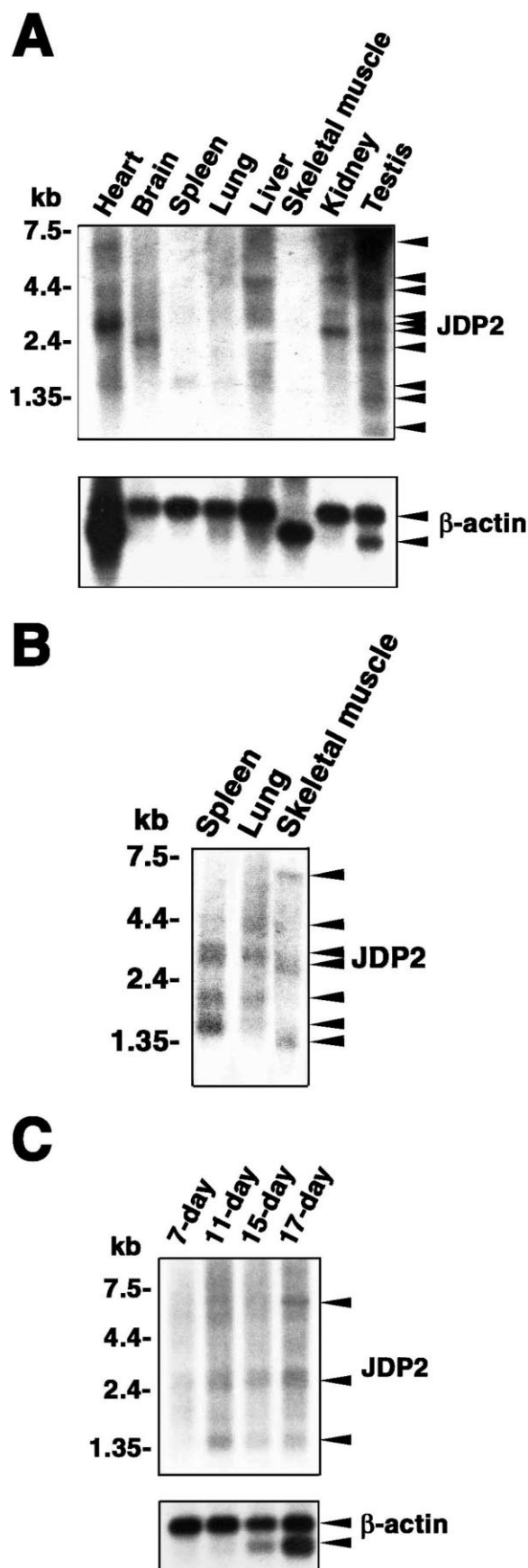
Fig. 3. Northern blotting analysis of JDP2 transcripts. (A) Transcripts on a mouse MTN blot filter were allowed to hybridize with a 1.5-kb ^{32}P-radiolabeled fragment of JDP2 cDNA. The radioactive probe was washed from the blot after autoradiography and the blot was reprobed with cDNA for β-actin. (B) The transcripts on a mouse MTN blot were allowed to hybridize with the JDP2 cDNA probe as indicated in (A) but exposure was extended for another 8 h. (C) The transcripts on a mouse embryo MTN blot were allowed to hybridize with the JDP2 cDNA probe. The transcripts on the filter were rehybridized with a cDNA for β-actin after removal of the JDP2 cDNA probe from the filter. The sizes of JDP2 transcripts are indicated on the left.
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which two nucleotides in the CRE had been changed. The expression of JDP2 had no effect on the ATF-2-mediated transcription of this altered mCRE/tk-CAT reporter gene (Fig. 4B). The results demonstrated that JDP2 specifically repressed the CRE-dependent transcription of the reporter construct that was mediated by ATF-2, with a decrease in the level of expression of the reporter of approximately 10-fold. To examine the contribution of JDP2 to ATF-2-mediated transactivation in further detail, we studied the effect of over-expression of JDP2 on a CRE-dependent reporter gene. As shown in Fig. 4C, over-expression of JDP2 suppressed expression of the reporter gene in a dose-dependent manner. By contrast, the coexpressing empty vector did not affect the reporter activity that was mediated by ATF-2.

3.5. DNA-binding activity of JDP2 in vitro

We examined the DNA-binding activity of JDP2 in vitro by an EMSA. GST-fused JDP2 and GST-fused ATF-2 were produced in *E. coli* and purified on a column of glutathione-Sepharose. The formation of DNA–protein complexes with a CRE probe in vitro was confirmed by the supershifting of bands that occurred when antibodies against ATF-2 or against GST were included in the reaction mixture. As shown in Fig. 5A, GST-fused JDP2 generated two distinct bands (lane 2) and the mobility of both bands was reduced, or the bands disappeared when antibodies specific for JDP2 or GST were included (data not shown). The rapidly migrating band might represent the complex of the DNA probe with GST-JDP2 that had lost the amino-terminal region of GST-JDP2 but still retained the intact carboxy-terminal region of GST-JDP2. GST by itself did not bind to the CRE probe (lane 1). The non-radiolabeled oligodeoxynucleotide that corresponded to CRE competed for binding to the GST-JDP2 fusion protein (lane 3). By contrast, a mutated oligodeoxynucleotide did not compete with radiolabeled CRE for binding to GST-JDP2 (lane 4). In similar analyses, we also detected the complex of the DNA probe with the GST-ATF-2 homodimer in vitro. We observed competition by non-radiolabeled CRE but not by non-radiolabeled mutated CRE (lanes 5–7).

To our surprise, we did not detect a significant DNA–protein complex with the GST-ATF-2/GST-JDP2 heterodimer in the presence of the CRE probe DNA (data not shown). It could be reasoned that the absence of significant retarded GST-ATF-2/GST-JDP2 complex is due to the structural hindrance of GST fusion. Thus, we next examined the formation of the JDP2/ATF-2 heterodimer by in vitro translated proteins. As shown in Fig. 5B, each homodimer of in vitro translated ATF-2 and of JDP2 bound to the CRE probe (lanes 2 and 3), but reticulocyte lysate failed to bind the CRE probe

by itself. The JDP2 also formed a heterodimer with the ATF-2 (lane 4) in the presence of the CRE probe, with a molecular weight different from that attributed to the ATF-2 or JDP2 homodimer. These data demonstrate that JDP2 binds to CRE as a homodimer or heterodimer with ATF-2.

4. Discussion

We report here the identification of mouse JDP2 as the interaction partner of ATF-2 in a yeast two-hybrid screening test. We also demonstrated that JDP2 negatively regulates the transcription of a CRE-dependent promoter that is mediated by ATF-2. In a binding assay in vitro (Fig. 2A), more than 10% of the input ATF-2 protein was associated with JDP2 and vice versa. In a pull-down assay in vivo, ATF-2 and histidine-tagged JDP2 were co-purified by TALON resins (Fig. 2B). Moreover, using mutant ATF-2 and JDP2, we determined the interaction domain between JDP2 and ATF-2 as their bZIP region. The DNA-binding assay also showed that JDP2 makes a heterodimer with ATF-2, although its formation is not a predominant one. A histidine-tagged derivative of JDP2 was localized in the nuclei of mouse fibroblasts and embryonal carcinoma cells, as is ATF-2 (data not shown). These results demonstrated that JDP2 interacted with ATF-2 both in vitro and in vivo in a classical way.

JDP2 transcripts were expressed in all mouse tissues examined, albeit at different levels. At least 10 transcripts were identified and abundant expression of JDP2 mRNAs was detected in the heart, brain, liver, kidney and testis, with less abundant expression in the spleen, lung and skeletal muscle. In each case, transcripts of different sizes were expressed. Southern blotting analysis indicated that JDP2 is encoded by a single-copy gene in the mouse genome (data not shown). It remains to be determined how the various transcripts are generated from the single gene. It is possible that alternative splicing might be responsible for this phenomenon. The level of JDP2 transcripts increased during embryonic development. Moreover, sequence analysis revealed very high conservation between the human and the rodent JDP2 proteins (Fig. 1B). These results imply that the expression of JDP2 is important or essential for development of the embryo, as well as for maintaining normal structure and/or function.

We examined the role of JDP2 in regulating the transcriptional activity of ATF-2. As expected, ATF-2 enhanced the expression of the CRE/tk-CAT reporter gene. Simultaneous expression of JDP2 repressed such expression. Aronhein et al. [32] reported that JDP2 repressed the activity of c-Jun to stimulate a *jun*-luciferase reporter and that it also repressed activation of the reporter construct by a combination of c-Jun
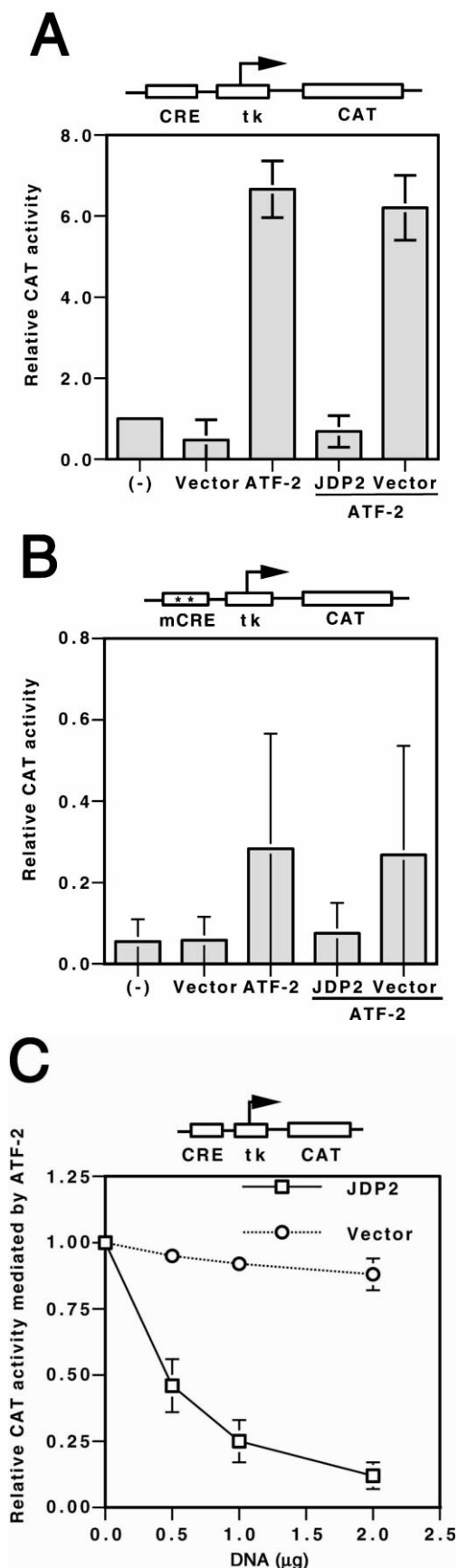

Fig. 4. JDP2-mediated effects on the transcriptional activity of ATF-2. NIH3T3 cells were cotransfected with (A) a CRE/tk-CAT reporter (0.2 μg) or (B) a mutant CRE/tk-CAT reporter (0.2 μg) together with either the empty vector or expression vectors for JDP2 and ATF-2 (pcDNA4-JDP2, pECE-ATF-2). At 24 h after transfection, cells were collected and CAT activity was determined. The data represent the means ± S.E.M. of results of three independent experiments, with the relative CAT activity for the wild-type CRE/tk-CAT reporter alone being normalized to 1.0. Note the different scales in (A) and (B). (C) The details of the experiment corresponded to those in (A) but increasing amounts of the expression vector that encodes JDP2, or the empty vector, were used.
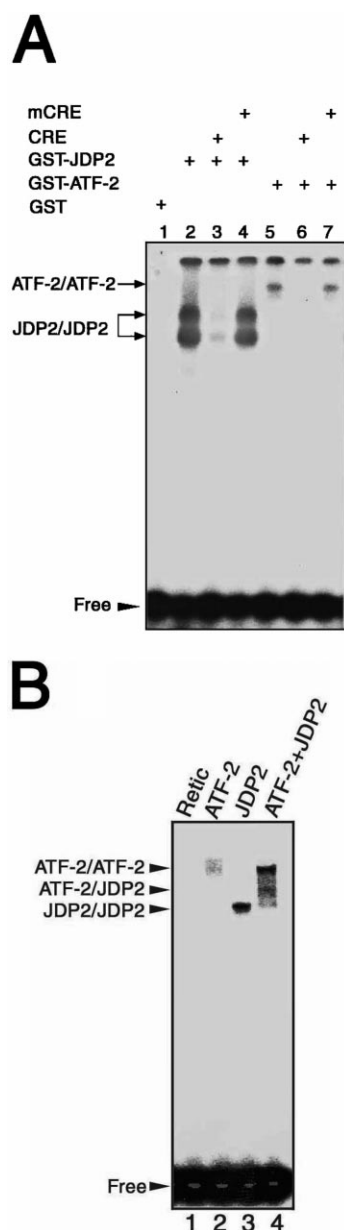
Fig. 5. DNA-binding activity of JDP2 and dimerization with ATF-2. (A) EMSA using GST-JDP2 and/or GST-ATF-2 and ^{32}P-radiolabeled CRE. The GST-fused JDP2 protein (or GST-ATF-2) was incubated with [^{32}P]CRE and then analyzed by EMSA. Binding to the radiolabeled CRE by GST-JDP2 (or GST-ATF-2) was eliminated by a 25-fold molar excess of unlabeled CRE (lanes 3 and 6) or the mutated mCRE (lanes 4 and 7). (B) An EMSA was performed with in vitro translated JDP2 and ATF-2. Retic, refers to unprogrammed reticulocyte lysate. The migration positions of the ATF-2/ATF-2 homodimer, ATF-2/JDP2 heterodimer and JDP2/JDP2 homodimer are indicated. 'Free' indicates the free DNA probe.

and c-Fos. Here we observed similar suppression of the ATF-2-dependent transcription of a CRE-controlled reporter by JDP2, as shown in Fig. 4.

The DNA-binding study indicated that JDP2 formed a stable homodimer, or a weak heterodimer, with ATF-2 and bound to CRE (Fig. 5). Aronhein et al. [32] reported that JDP2 formed a strong heterodimer with c-Jun in the presence of TRE, thereby enhancing the DNA-binding activity of c-Jun. We do not know the exact reasons for this difference. It

should be considered that the differential DNA-binding affinity of JDP2 in homodimers or heterodimers might depend on the partner in the dimerization and on the DNA sequence involved in the regulation of transcription. Aronhein et al. [32] also proposed that JDP2 might repress the TRE-dependent transcriptional activity of c-Jun by forming a c-Jun/JDP2 heterodimer, that competes with activators for binding to c-Jun. Alternatively, JDP2 also enhances the binding of c-Jun to TRE, thereby providing further inhibition by competition with active heterodimers for binding to the TRE. Because JDP2 did not make a strong heterodimer with ATF-2, as with c-Jun, it seems likely that neither of the above two mechanisms is the major one, by which JDP2 might repress ATF-2-mediated CRE-controlled transcription. One possible mechanism might be that JDP2 represses ATF-2-mediated transcriptional activity by recruiting some inhibitory factors, such as histone deacetyltransferases, to the CRE promoter. Chen et al. [36] reported that ATF3 represses transcription from the promoters with ATF sites by stabilizing the inhibitory co-factors at the promoter. The high sequence identity between JDP2 and ATF3 might suggest the similar mechanisms of the transcriptional repression. These problems remained to be solved.

The results of DNA-binding and reporter assays suggest that JDP2, a ubiquitously expressed bZIP protein, plays a key role in the regulation, not only of Ap-1/TRE-dependent transcription, but also of CRE-dependent transcription. Thus, JDP2 might be a general repressor of transcription, that is related to the Jun/Fos/ATF-2 family. The fact that JDP2 is a repressor of ATF-2 implies that it might inhibit RA-mediated transcription of the *c-jun* gene in F9 cells [21]. Our initial purpose was to isolate a *c-jun* activator within the DRF complexes [21], so we are now not only studying the effect of JDP2 on the differentiation of F9 cells, but also analyzing which one, among the proteins isolated as ATF-2 interaction partners in this study, was included in the DRF complexes and activates the *c-jun* transcription in F9 cells.

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