Drosophila Activating Transcription Factor-2 Is Involved in Stress Response via Activation by p38, but Not c-Jun NH₂-Terminal Kinase

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Activating transcription factor (ATF)-2 is a member of the ATF/cAMP response element-binding protein family of transcription factors, and its *trans*-activating capacity is enhanced by stress-activated protein kinases such as c-Jun NH₂-terminal kinase (JNK) and p38. However, little is known about the *in vivo* roles played by ATF-2. Here, we identified the *Drosophila* homologue of ATF-2 (dATF-2) consisting of 381 amino acids. In response to UV irradiation and osmotic stress, *Drosophila* p38 (dp38), but not JNK, phosphorylates dATF-2 and enhances dATF-2-dependent transcription. Consistent with this, injection of dATF-2 double-stranded RNA (dsRNA) into embryos did not induce the dorsal closure defects that are commonly observed in the *Drosophila* JNK mutant. Furthermore, expression of the dominant-negative dp38 enhanced the aberrant wing phenotype caused by expression of a dominant-negative dATF-2. Similar genetic interactions between dATF-2 and the dMEKK1–dp38 signaling pathway also were observed in the osmotic stress-induced lethality of embryos. Loss of dATF-2 in *Drosophila* S2 cells by using dsRNA abrogated the induction of 40% of the osmotic stress-induced genes, including multiple immune response-related genes. This indicates that dATF-2 is a major transcriptional factor in stress-induced transcription. Thus, dATF-2 is critical for the p38-mediated stress response.

INTRODUCTION

The activating transcription factor/cAMP response element-binding protein (ATF/CREB) family of proteins bears a DNA-binding domain consisting of a cluster of basic amino acids and a leucine zipper that together form the so-called b-ZIP structure. These proteins can form homodimers or heterodimers by binding via their leucine zipper motifs, after which they can bind to the cyclical AMP response element (CRE: 5'-TGACGTCA-3') via their basic region (Hai and Curran, 1991). The two major subgroups of the ATF/CREB family proteins are CREB and ATF-2 (originally designated as CRE-BP1) (Gonzalez and Montminy, 1989; Maekawa *et al.*, 1989). The CREB subgroup includes CREB and cAMP response element modulator (CREM) (Gonzalez and Montminy, 1989; Foulkes *et al.*, 1991), whereas the ATF-2 subgroup contains ATF-2 (Hai *et al.*, 1989; Maekawa *et al.*, 1989), ATFa (recently also called ATF-7) (Gaire *et al.*, 1990), and CRE-BPa (Nomura *et al.*, 1993). When the Ser-133 residue of CREB is phosphorylated by cAMP-dependent protein kinase, CREB can bind to the transcriptional coacti-

vator CREB-binding protein (CBP), which greatly stimulates the *trans*-activating capacity of CREB (Chrivia *et al.*, 1993). The *trans*-activating capacity of ATF-2, on the other hand, is enhanced by the phosphorylation of its Thr-69 and Thr-71 residue by stress-activated protein kinases (SAPKs) such as c-Jun NH₂-terminal kinase (JNK) and p38 (Gupta *et al.*, 1995; Livingstone *et al.*, 1995; van Dam *et al.*, 1995). SAPKs are activated by various extracellular stress such as UV, osmotic stress, and inflammatory cytokines. All three members of the ATF-2 subgroup bear the *trans*-activation domain in their N-terminal region: this domain consists of two subdomains, namely, the N-terminal subdomain containing the well known zinc finger motif and the C-terminal subdomain containing the SAPK phosphorylation sites (Matsuda *et al.*, 1991; Nagadoi *et al.*, 1999). The latter subdomain has a highly flexible and disordered structure. Although the coactivator CBP binds to the protein surface of b-ZIP domain of ATF-2 (Sano *et al.*, 1998), the cofactor that binds to the N-terminal activation domain of ATF-2 remains unknown.

The physiological roles played by ATF-2 have been analyzed by using mutant mice. Null *Atf-2* mutant mice die shortly after birth and display symptoms of severe respiratory distress and have lungs filled with meconium (Maekawa *et al.*, 1999). In the mutant embryos, hypoxia occurs, which may lead to strong gasping respiration with the consequent aspiration of the amniotic fluid containing meconium. This is due to the impaired development of cytotrophoblast cells in the placenta that in turn is caused by decreased levels of expression of the platelet-derived growth factor receptor α . In addition, another *Atf-2* mutant mouse, which expresses only a fragment of ATF-2, exhibits lowered postnatal viability and growth, a defect in endochondrial

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Abbreviations used: dATF-2, *Drosophila* ATF-2; dp38, *Drosophila* p38; dsRNA, double-stranded RNA; JNK, Jun NH₂-terminal protein kinase; MAPK, mitogen-activated protein kinase; SAPKs, stress-activated protein kinases.

ossification, and reduced numbers of cerebellar Purkinje cells (Reimold *et al.*, 1996). However, the physiological roles played by the other ATF-2 family proteins remain unknown.

Because *Drosophila* has a low degree of gene redundancy and therefore fewer related genes compared with mammals, it is sometimes advantageous to analyze the *Drosophila* homologue of a mammalian gene of interest when trying to determine its function. Moreover, if appropriate *Drosophila* mutants are available, a variety of genetic experiments can be performed to identify the *in vivo* function of the gene in question. In *Drosophila*, three members of the mitogen-activated protein kinase (MAPK) protein family have been identified: Rolled (Erk homologue), dJNK (JNK homologue, also called Basket), and dp38a and dp38b (p38 homologue). Rolled mediates various receptor tyrosine kinase signals in the process of tracheal elaboration, cell proliferation, mesodermal patterning, R7 photoreceptor cell differentiation, and differentiation of terminal embryonic structures (Biggs *et al.*, 1994; Brunner *et al.*, 1994; Gabay *et al.*, 1997a,b). On the other hand, the pathway containing Hemipterous (Hep; MAPK kinase [MAPKK] homologue), dJNK, and *Drosophila* Jun (dJun) is involved in dorsal closure during embryo development (Glise *et al.*, 1995; Glise and Noselli, 1997; Hou *et al.*, 1997; Riesgo-Escovar *et al.*, 1996; Sluss *et al.*, 1996; Riesgo-Escovar and Hafen, 1997). All mutants of this pathway exhibit the dorsal open phenotype and a decreased level of the expression of Decapentaplegic (Dpp), a secretory ligand belonging to the transforming growth factor (TGF)- β superfamily, in leading edge cells. With regard to the dp38s, they are phosphorylated by various stresses, including UV, lipopolysaccharide (LPS), and osmotic stress (Han *et al.*, 1998a,b). The phenotype resulting from the ectopic expression of the dominant negative (DN) dp38b in the wing imaginal disc indicates that dp38b functions downstream of thickvein (Tkv), a type I receptor of the Dpp ligand, in wing morphogenesis (Adachi-Yamada *et al.*, 1999).

To determine the *in vivo* function of ATF-2, we identified and characterized the *Drosophila* ATF-2 homologue (dATF-2). These studies are reported here and show that dATF-2 is directly phosphorylated by dp38b but not by dJNK. Moreover, genetic analyses indicated that dATF-2 acts in the dp38 signaling pathway. In addition, DNA array analysis demonstrated that dATF-2 is a major transcriptional activator of osmotic stress-inducible genes.

MATERIALS AND METHODS

Isolation of a dATF-2 cDNA Clone

The *Drosophila* database was searched for a protein sequence that is homologous to the N-terminal 150 amino acids of human ATF-2, and the corresponding DNA fragment was amplified by PCR from the *Drosophila* embryonic cDNA library (Novagen, Madison, WI). Using this amplified fragment as a probe, the cDNA clone encoding dATF-2 was isolated from the same cDNA library.

Detection of Endogenous dATF-2

Schneider S2 cells were cultured at 25°C in the Schneider's *Drosophila* medium (Invitrogen, Carlsbad, CA) supplemented with 10% of fetal bovine serum. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 1 mM β -glycerophosphate, 25 mM NaF, and protease inhibitor cocktail) on ice for 10 min. Clarified lysate were collected by centrifugation, and 200 μ g of total lysate protein was resolved by SDS-PAGE by using 4–12% gradient gel, followed by Western blotting by using the rabbit anti-dATF-2 antibody that was raised against *Pseudomonas* toxin full-length dATF-2 fusion protein. To exogenously express dATF-2, the plasmid expressing N-terminally FLAG-tagged dATF-2 was constructed by inserting the dATF-2 cDNA downstream of the *Drosophila* actin 5C promoter (Thummel *et al.*, 1988). S2 cells were transfected with the dATF-2 expression plasmid by using CaPO₄ method. Two days later, the lysates were prepared

as described above, and 10 μ g of total lysate protein was used for Western blotting. To produce the dATF-2 double-stranded RNA (dsRNA), the 300-base pair *EcoRI-PstI* fragment of the dATF-2 cDNA was inserted into pBlue-script II vector. Both strands of the dATF-2 cDNA fragment were transcribed *in vitro* by using T7 or T3 RNA polymerase and then annealed. For RNA interference experiment, 4×10^6 of S2 cells were plated in 3 ml of serum-free medium. dsRNA (50 μ g) was added directly to the medium, and the cells were rocked for 30 min at room temperature. Seven milliliters of medium supplemented with 10% fetal calf serum was added, and the cells were incubated at 25°C for 4 d, and cell lysates were prepared as described above.

Dimer Formation Assays

Glutathione S-transferase (GST) pull-down assays with various mutants of GST-dATF-2 and *in vitro*-translated full-length dATF-2 were performed essentially as described previously (Dai *et al.*, 1996). The binding buffer contained 20 mM HEPES, pH 7.7, 150 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl₂, 1% skim milk, 1 mM dithiothreitol (DTT), and 0.05% NP-40.

Gel Shift Assays

The gel shift assay was performed essentially as described previously (Maekawa *et al.*, 1989). Briefly, ~0.1–0.4 μ g of purified GST-dATF-2 proteins containing various regions of dATF-2 was incubated for 1 h at 25°C with a ³²P-labeled oligonucleotide in a solution containing 10 mM Tris-HCl, pH 7.9, 50 mM KCl, 1 mM DTT, 0.04% NP-40, 1 μ g of poly(dI-dC), and 5% glycerol. The reaction mixture was separated by a 4% PAGE in 0.5 $\times$ TGE buffer (25 mM Tris-HCl, 19 mM glycine, and 1 mM EDTA). This was followed by autoradiography. The sequences of oligonucleotides used as probes are as follows: 5'-TCGGGAAATGACGTCATCTCCAGC-3' (ATF/CRE) (Hai *et al.*, 1989) and 5'-TCGGGAAATGAAGTGATCTCCAGA-3' (mutant ATF/CRE) (Hai *et al.*, 1989).

In Vitro Kinase Assay

Bacterial lysates containing GST-dJNK, GST-dp38b, GST-HEP, GST-dMKK3, and various forms of GST-dATF-2 were prepared and mixed with glutathione-Sepharose beads. After washing, the bound proteins were eluted with elution buffer (100 mM Tris-HCl, pH 8.0, 20 mM glutathione, and 120 mM NaCl) and dialyzed overnight in kinase buffer (20 mM HEPES, pH 7.4, 20 mM MgCl₂, 25 mM β -glycerophosphate, 0.1 mM Na₂VO₄, and 2 mM DTT). Approximately 1 μ g of kinase protein and 1 μ g of GST-dATF-2 were incubated for 1 h at 30°C in kinase buffer in the presence of [γ -³²P]ATP. The proteins were then analyzed by SDS-PAGE followed by autoradiography.

In Vivo Kinase Assays

S2 cells were transfected with the FLAG-dATF-2 expression plasmid by using CaPO₄ method. Two days later, the cells were irradiated by UV (100 J/m²) or treated with 0.5 M sorbitol for 15 min and further cultivated for various times. The cells were then disrupted by using RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 25 mM NaF, 25 mM β -glycerophosphate, and 0.1 mM Na₂VO₄) containing a protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany), and the lysates were subjected to SDS-PAGE, followed by Western blotting with an anti-FLAG monoclonal antibody (mAb) (M5; Sigma-Aldrich, St. Louis, MO). For the phosphatase treatment, cell lysates were prepared using the lysis buffer (50 mM HEPES, pH 7.5, 250 mM NaCl, 0.2 mM EDTA, and 0.5% NP-40) containing a protease inhibitor, and FLAG-dATF-2 was immunoprecipitated with the anti-FLAG antibody. The immunocomplexes were then suspended with phosphatase buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 2 mM MnCl₂, 5 mM DTT, and 0.01% Brij35) and incubated with λ -phosphatase for 30 min at 16°C. The reaction was terminated by adding SDS-phosphate buffer. The proteins were analyzed by Western blotting with a rabbit antibody raised against GST-dATF-2(1-150).

In Vivo Labeling of dATF-2

S2 cells were transfected with the FLAG-dATF-2 expression vector and labeled with [³²P]orthophosphate for 4 h. Lysates were prepared from the transfected cells using lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 4 mM EDTA, 1 mM DTT, 10 mM Na₂P₂O₇, 10 mM NaF, 2 mM Na₂VO₄, and protease inhibitor), and the dATF-2 proteins were immunoprecipitated with anti-FLAG antibody, and analyzed by SDS-PAGE, followed by autoradiography.

Luciferase Reporter Assays

The GAL4-dATF-2 expression plasmid was constructed by a PCR-based method by using the cytomegalovirus promoter-containing vector pCMX (Umesono *et al.*, 1991). The luciferase reporter (3GAL-TK-luc; 1 μ g), in which three copies of GAL4-binding site and the firefly luciferase gene are linked to the thymidine kinase promoter, was transfected into S2 cells together with the GAL4-dATF-2 expression plasmid (1, 2, or 4 μ g) and the internal control plasmid pRL-CMV (0.2 μ g) by the CaPO₄ method. The total amount of plasmid DNA introduced was adjusted to 10 μ g with the empty vector.

Luciferase activities were measured by using the dual luciferase assay system (Promega, Madison, WI). In the experiments using the CRE-containing reporter (4CRE-Adh-luc), in which four copies of ATF/CRE sites and the firefly luciferase gene are linked to the *Drosophila* alcohol dehydrogenase gene promoter, a mixture of 1 μ g of the reporter, 0.5 μ g of the dATF-2 expression plasmid, and 0.2 μ g of pRL-CMV was transfected into S2 cells. To examine the effect of dJNK/Hep or dp38/dMKK3 on dATF-2-induced transcriptional activation, a mixture of 4CRE-Adh-luc reporter (1 μ g), pact5C-FLAG-dATF-2 (1.5 μ g), pact5C-HA-dJNK or pact5C-HA-dp38b (1 μ g), pact5C-Hep or pact5C-dMKK3 (0.5 or 2 μ g), and the internal control plasmid pact5C-retinaluc (50 ng) was transfected into S2 cells. Alternatively, a mixture of 3GAL4-TK-luc reporter (1 μ g), pCMV-GAL4-dATF-2 or pCMV-GAL4-dJUN (2 μ g), pact5C-HA-dJNK or pact5C-HA-dp38b (0.5 μ g), pact5C-Hep or pact5C-dMKK3 (0.5 or 2 μ g), and pact5C-retinaluc (50 ng) was transfected into S2 cells. In both cases, the total amount of plasmid DNA was adjusted to 10 μ g with the empty vector. To examine the effect of SB203580 on the dATF-2 induced transcriptional activation, 1 μ g of 4CRE-Adh-luc reporter was transfected into S2 cells together with 1 μ g of pact5C-FLAG-dATF. Twenty-four hours after transfection, SB203580 was added to the medium at a final concentration of 0.4, 2, 5, or 10 μ M.

In Situ Hybridization

Wild-type embryos ranging from 0 to 16 h old were prepared and in situ hybridization was carried out essentially as described by Han *et al.* (1998b). Riboprobes of dATF-2 were labeled by using a digoxigenin RNA labeling kit (Roche Diagnostics) and hybridized at 45°C. The probes were detected with a mAb against digoxigenin coupled to alkaline phosphatase (ALP) and BM purple as a substrate (Roche Diagnostics).

Injection of dsRNA

The dATF-2 dsRNA RNA precipitates were dissolved in injection buffer (5 mM KCl and 0.1 mM Na₂HPO₄, pH 6.8) to a final concentration of 10 μ M. Embryos were collected over a 30–50-min period at 25°C, dechorionated (in 25% NaClO), and attached to a coverslip with double stick tape. The embryos were then desiccated and covered with Voltaef H 10S oil (Elf Atochem, Paris, France). Most embryos were injected at the precellular blastoderm stage. The location of the injection was on the ventral side (15–80% egg length). Cuticle preparation was performed according to a standard protocol except that the embryos were not fixed before mounting.

Fly Strains

Drosophila cultures and crosses were carried out by standard procedures at 25°C. The DNA fragment encoding the C-terminal 152 amino acids (amino acids 230–381) of dATF-2 was cloned into pUAST, a GAL4-responsive vector (Brand and Perrimon, 1993), to generate pUAST-dATF-2²³⁰⁻³⁸¹ (DN-dATF-2). To test for modification of the DN-dATF-2 phenotype, a transgenic strain carrying pUAST-D-MEKK1a or pUAST-p38b^{DN} (Inoue *et al.*, 2001) was used. *bsk¹* is a null mutant of the *bsk* (dJNK) gene (26).

Sensitivity to Osmotic Stress

Parent flies were allowed to lay eggs every day while feeding on a diet containing the indicated concentrations of NaCl. The viability was calculated from the number of adult flies per the number of first larvae derived from mating *hs-GAL4* females with upstream activating sequence (UAS) transgenic males.

DNA Microarrays

S2 cells were plated on a 10-cm dish to achieve 10% confluence, and a mixture of 1 μ g of the dATF-2 dsRNA; 2 μ g of the pAct5C-EGFP plasmid, in which the *Drosophila* actin 5C promoter was linked to the enhanced green fluorescent protein (EGFP) cDNA; and 13 μ g of the empty control vector pAct5C0 was transfected by the CaPO₄ method. Two days after transfection, the cells were treated with 0.5 M sorbitol for 15 min, and further cultivated for 3 h. The cells expressing EGFP were obtained by cell sorting using FACSsort (BD Biosciences, San Jose, CA). Immediately after sorting, the cells were mixed with TRIzol reagent (Invitrogen), and biotin-labeled RNA for GeneChip analysis was then prepared according to the GeneChip eukaryotic small sample target labeling assay version II protocol, which is available on the Affymetrix Web site at http://www.affymetrix.com/support/technical/technote/smallv2_technote.pdf. The *Drosophila* genome array that contains >13,500 probe sets (Affymetrix) was used for the assay.

RESULTS

Identification of dATF-2

To identify the *Drosophila* gene that bears homology to the N-terminal 150-amino acid region of human ATF-2, which contains the JNK/p38 phosphorylation sites, we searched the *Drosophila* expressed sequence tag (EST) database. One

EST clone was significantly homologous to this sequence (59% identity, 68% similarity). We amplified this clone from the *Drosophila* embryonic cDNA library and used it to probe the same library to obtain the full-length cDNA clone. The isolated clone was 1.35 kb in length, contained a poly(A) tail at its 3' end, and had an open reading frame of 1143 base pairs corresponding to 381 amino acid residues (Figure 1A). In Northern blotting analysis with RNA from *Drosophila* Schneider S2 cells, the isolated cDNA probe hybridized with a single mRNA species of ~1.35 kb (Figure 1B). The protein encoded by this clone is significantly homologous to human ATF-2. Thus, we designated this protein as dATF-2. The homology between dATF-2 and human ATF-2 was found in three regions, namely, the N-terminal region around the SAPK phosphorylation sites, the basic region, and the leucine zipper region (59, 40, and 35% identity, respectively) (Figure 1C). dATF-2 also has the similar degree of homology with human ATF α and CRE-BP α in these two regions, because three members of ATF-2 subfamily (ATF-2, ATF α , and CRE-BP α) have a high homology in these regions. The entire regions of the two proteins share 39% identity and 47% similarity. The dATF-2 gene, which is located at 2R-60E4, consisted of two exons (Figure 1D). However, the database of the Berkley *Drosophila* Genome Project contains a cDNA sequence denoted as CG30420-PA (Figure 1D) that only contains the first exon of dATF-2 and an additional 2.0-kb region. However, we could not detect 3.3-kb mRNA by using the dATF-2 probe, which suggests that this cDNA may have been artificially generated during the cDNA cloning.

To confirm that this dATF-2 protein is really expressed in vivo, we prepared the rabbit polyclonal antibody against GST-dATF-2 fusion protein and used it to detect endogenous dATF-2. This antibody detected the 57-kDa protein in Western blotting by using whole cell lysates prepared from Schneider S2 cells (Figure 1E, lane 1), which has a similar size with exogenously expressed dATF-2 in transfected S2 cells (Figure 1E, lane 4). Treatment of cells with osmotic stress (0.5 M sorbitol) caused the slow migration of both endogenous and exogenous dATF-2 (Figure 1E, lanes 2 and 5), suggesting that dATF-2 is phosphorylated by osmotic stress. We also confirmed that dATF-2 dsRNA transfection markedly decreased the levels of both endogenous and exogenous dATF-2 (Figure 1E, lanes 3 and 6). Thus, dATF-2 identified here is really expressed in vivo.

Formation of dATF-2 Dimers and Their Binding to CRE

We examined the capacity of dATF-2 to form homodimers and heterodimers. Thus, various GST-dATF-2 fusion proteins, which consist of the amino acids 152–381 of dATF-2 with various mutations of the leucine zipper (Figure 2A, left), were prepared and mixed with the in vitro-translated full-length dATF-2. The in vitro-translated dATF-2 efficiently bound to the wild-type GST-dATF-2, which indicates that dATF-2 forms a homodimer (Figure 2A, right). Mutations of the C-terminal two leucines into valines (L34V) did not abrogate homodimer formation. However, when the same leucines were replaced with prolines (L34P), or when all four leucines were replaced by valine (L1234V), or when the whole leucine zipper region was deleted (Δ LZ), GST-dATF-2 failed to bind. Thus, the leucine zipper structure is critical in the formation of a dATF-2 homodimer, which is also true for mammalian ATF-2 (Matsuda *et al.*, 1991). Next, we tested whether the N-terminal portion of dATF-2 can intramolecularly interact with its C-terminal region (Figure 2B). GST pull-down assays showed that the in vitro-translated dATF-2 bound efficiently to GST fusion protein con-

Figure 1. Structure of the dATF-2 gene. (A) Amino acid sequence deduced from the nucleotide sequence of the dATF-2 cDNA. The putative SAPK phosphorylation sites, and the basic amino acids and leucines in the b-ZIP region are shown by bold letters. The GenBank accession number for the dATF-2 cDNA sequence is AY956383. (B) Northern blot analysis of dATF-2 mRNA. Poly(A)⁺ RNA from S2 cells (10 μ g) was subjected to Northern blotting with the dATF-2 probe. (C) Comparison of the dATF-2 and human ATF-2 sequences. (Top) Structures of dATF-2 and human ATF-2 are shown schematically. The JNK/p38 phosphorylation sites and the DNA-binding domain containing the b-ZIP structure are indicated. The percentages of homology between each of the domains are indicated. (Bottom) Comparison of the sequences of dATF-2 and human ATF-2 domain counterparts. The amino acids that are identical and conserved are indicated by lines and dots, respectively. (D) Genomic organization of the dATF-2 gene. The exons are indicated by boxes. The shaded and open boxes are the translated and untranslated regions, respectively. The CG30420-PA cDNA clone, which was deposited in the Berkeley *Drosophila* Genome Project database, may be an artificial clone. (E) Detection of endogenous dATF-2. Total cell lysates were prepared from S2 cells treated with sorbitol or dATF-2 ds-RNA or from control cells, and used for Western blotting with anti-dATF-2 antibody. As a control, cell lysates prepared from the S2 cells transfected with the dATF-2 expression plasmid were also used (lanes 4–6). Asterisk indicates the nonspecific band.

taining the C-terminal half of dATF-2, but not to the GST fusion containing its N-terminal half.

To determine whether dATF-2 can bind to the same DNA sequence as mammalian ATF-2, we prepared three GST-dATF-2 fusion proteins containing various regions of dATF-2 and used them in gel retardation assays (Figure 2, C and D). The DNA probe used contained the artificial sequence derived from the adenovirus early E1aE promoter that has the consensus CRE (ATF/CRE probe) (Hai *et al.*, 1989). All three GST-dATF-2 fusion proteins formed protein–DNA complexes whose mobility depended on their molecular weights, whereas the probe was not retarded by the control GST protein (Figure 2D). These results indicate that a dATF-2 homodimer binds to the consensus CRE sequence. To confirm the specificity of sequence recognition by

dATF-2, a mutated CRE probe containing two base mutations in the CRE sequence was used. GST-dATF-2 was not able to bind to this mutant probe in gel shift assays (Figure 2E, left). Furthermore, in competition experiments, this mutant DNA did not block the binding of dATF-2 to CRE (Figure 2E, right). Thus, dATF-2 specifically binds to the CRE.

In Vitro Phosphorylation of dATF-2 by dp38

To investigate whether, like mammalian ATF-2, dATF-2 can be phosphorylated by SAPKs, we performed in vitro kinase assays by using recombinant proteins. GST-dATF-2 or GST-dJun served as the substrate, whereas the GST fusion containing dp38b or dJNK acted as the kinase. These proteins were mixed and then incubated with [γ -³²P]ATP. Interestingly, dATF-2 was phosphorylated by dp38b, but not by

Figure 2. Binding of a dATF-2 homodimer to the CRE sequence. (A) Homodimer formation of dATF-2 via its leucine-zipper structure. Various forms of GST-dATF-2 fusion proteins that contain the 152–381 amino acid region of dATF-2 with the mutated leucine-zipper structures indicated on the left were prepared. Binding of in vitro-translated full-length wild-type dATF-2 to these GST-dATF-2 proteins was examined by GST pull-down assays. The amount of dATF-2 in the input lane was 10% of that used for the binding assay. (B) No intramolecular interaction of dATF-2. (Left) GST fusion proteins containing the N- or C-terminal region of dATF-2 were expressed in *Escherichia coli*, purified, and analyzed by SDS-PAGE, followed by Coomassie staining. (Right) GST pull-down assays were performed with in vitro-translated full-length dATF-2 and the GST fusion proteins indicated above each lane. (C) Structure of the GST-dATF-2 proteins used for the DNA-binding assays. (Left) Three GST fusion proteins containing various portions of dATF-2 are shown schematically. (Right) The proteins were expressed in *E. coli*, purified, and analyzed by SDS-PAGE, followed by Coomassie staining. (D) Binding of dATF-2 to the CRE. Gel retardation assays were performed using the probe containing the CRE derived from the adenovirus early E1aE gene along with different amounts of the GST-dATF-2 fusion proteins indicated above the gel. (E) Specific binding of dATF-2 to CRE. (Left) The probe containing the typical CRE derived from the adenovirus early E1aE promoter or its mutant was incubated with the GST-dATF-2 (246–381). (Right) DNA-binding reactions were performed as in the left panel except for the addition of a 10-, 100-, and 500-fold molar excess of the wild-type and mutant ATF/CRE oligonucleotides as competitors.

dJNK (Figure 3A). However, dJun was phosphorylated by both dp38b and dJNK to a similar degree. In mammals, the reverse is true because ATF-2 is phosphorylated by both p38 and JNK, whereas Jun is phosphorylated only by JNK (Han *et al.*, 1998b).

Based on its homology to vertebrate ATF-2, it is likely that the Thr-59 and Thr-61 residues of dATF-2 are dp38 phosphorylation sites. To investigate this, either or both residues were substituted with alanine (T59A, T61A, and T59/61A), and the resulting proteins were used in in vitro kinase assays with GST-dp38b (Figure 3B). The T59A and T61A mutants were only weakly phosphorylated by GST-dp38b, and the T59/61A mutant was not phosphorylated at all. Thus, dp38b directly phosphorylates dATF-2 at Thr-59 and Thr-61.

dp38 and dJNK are phosphorylated and up-regulated by their upstream kinases in their signaling pathways. These kinases are dMKK3 (*Drosophila* MAPKK 3) and Hep, respectively. To examine the effect of these MAPKKs on dATF-2 phosphorylation by dp38b and dJNK, recombinant GST-dMKK3 and GST-Hep were prepared and added to in vitro kinase assay mixtures containing dATF-2 and dp38b or dJNK (Figure 3C, left). As expected, the level of dp38b-

induced phosphorylation of dATF-2 was significantly increased when GST-dMKK3 was present. In contrast, dATF-2 was not phosphorylated at all by GST-dJNK, even if GST-Hep was present. In addition, we found that dp38a can phosphorylate dATF-2 and that this is enhanced by dMKK3 (Figure 3C, right). Thus, dATF-2 is a nuclear target of the dMKK3–dp38 pathway but not the Hep–dJnk pathway.

Stress-induced Phosphorylation of dATF-2

We next studied whether dATF-2 is phosphorylated by p38 in vivo in response to various stresses. Thus, *Drosophila* S2 cells were transfected with the FLAG-dATF-2 expression plasmid, exposed to UV irradiation or osmotic stress induced by 0.5 M-sorbitol, and then the phosphorylated dATF-2 was detected by Western blotting (Figure 3D). The phosphorylated dATF-2 bands showing slow migration occurred 15 min after UV irradiation or the addition of sorbitol, and they continued to be detected until 60 min after the stress treatment. Treatment of the cell extracts with λ -phosphatase converted nearly all of the slowly migrating bands into faster migrating bands (Figure 3E, left), which confirms

Figure 3. Phosphorylation of dATF-2 by dp38 in response to stress. (A) dp38b phosphorylates dATF-2 in vitro. Purified GST-dATF-2 (1-150) (top) or GST-dJun (bottom) was incubated in kinase buffer with the proteins indicated above each lane and [γ - 32 P]ATP. The proteins were separated by SDS-PAGE and then subjected to autoradiography. (B) Sites of dATF-2 that are phosphorylated by p38b. Wild-type or mutated GST-dATF-2(1-150) containing the mutation(s) shown above each lane, or the negative control GST-dATF-2(152-381) was analyzed by SDS-PAGE, followed by Coomassie Blue staining (top). These proteins were used in in vitro phosphorylation assays with GST-dp38b or GST (bottom). (C) dMKK3 enhances the dp38-dependent phosphorylation of dATF-2. GST-dATF-2(1-150) was used in the in vitro phosphorylation assay with the proteins shown above the lanes (left). GST fusions containing various kinases which were used in the phosphorylation assay were analyzed by SDS-PAGE, followed by Coomassie Blue staining (right). (D) In vivo phosphorylation of dATF-2 in response to stress. S2 cells were transfected with the FLAG-dATF-2 expression plasmid and treated with UV (left) or sorbitol (right). At the indicated time after stress treatment, the cell lysates were prepared and used in Western blotting with an anti-FLAG antibody. (E) In vivo phosphorylation of dATF-2 at Thr-59 and Thr-61 in response to stress. (Left) S2 cell lysates containing phosphorylated dATF-2 were prepared as described in D and immunoprecipitated with the anti-FLAG antibody. The immunocomplexes were then treated with λ -phosphatase (+) or control buffer (-) and subjected to Western blotting with the anti-dATF-2 antibody. (Right) Experiments were performed as described in D by using the plasmid that expresses wild-type dATF-2 or the mutant dATF-2 whose Thr-59 and Thr-61 residues have been mutated into Ala. (F) Effect of the p38 inhibitor on the dATF-2 phosphorylation. S2 cells were transfected with the FLAG-dATF-2 expression plasmid, treated with SB203580 at a final concentration of 1, 5, or 10 μ M, and then stimulated by sorbitol. Phosphorylated dATF-2 was detected as described above. (G) Effect of overexpressing dp38 (with or without dMKK3) or DN-p38 on in vivo dATF-2 phosphorylation. S2 cells were transfected with the FLAG-dATF-2 expression plasmid together with the plasmids that express the proteins indicated above the gel. dp38b AGF is a mutant dp38b protein whose dMKK3 phosphorylation sites, Thr-183 and Thr-185, have been mutated to Ala. Phosphorylated dATF-2 was detected as described above. (H) In vivo phosphorylation of dATF-2. S2 cells were transfected with FLAG-tagged wild type or T59/61A mutant of dATF-2 expression vector. Cells were labeled with [32 P]orthophosphate, and dATF-2 proteins were immunoprecipitated, analyzed by SDS-PAGE, and detected by autoradiography. In some case, cells were treated with sorbitol or SB203580.

that the retarded bands correspond to the phosphorylated forms of dATF-2. We next examined whether Thr-59 and Thr-61, both of which are phosphorylated by dp38b in vitro, were phosphorylated in vivo in response to the stress. When S2 cells were transfected with the plasmid that expresses the T59/61A mutant of FLAG-dATF-2, no slowly migrating bands were detected, even after UV irradiation and osmotic stress (Figure 3E, right). Thus, Thr-59 and Thr-61 are phosphorylated in vivo in response to stress.

To investigate whether dp38 is the kinase that phosphorylates dATF-2 in vivo, we used the imidazole compound SB203580 (Lee *et al.*, 1994), a mammalian p38-specific inhibitor. SB203580 cannot inhibit JNK activity but inhibits not only mammalian p38 but also dp38 (Han *et al.*, 1998). We found that SB203580 inhibited the osmotic stress-induced in vivo phosphorylation of dATF-2 in a dose-dependent manner (Figure 3F), which indicates that dp38 is a major kinase that phosphorylates dATF-2 in vivo in response to stress. To

Figure 4. dATF-2 is a transcriptional activator. (A) Transcriptional activation of the GAL4 site-containing luciferase reporter by the GAL4-dATF-2 fusion protein. (Top) Structures of the GAL4-dATF-2 fusion proteins used are shown schematically. The results of the *trans*-activation assays shown below are summarized on the right. (Bottom) S2 cells were transfected with the GAL4 site-containing luciferase reporter along with a plasmid expressing a GAL4-dATF-2 protein or control GAL4. Luciferase assays were then performed. The averages and standard deviations of three experiments are indicated. (B) Transcriptional activation of the CRE-containing luciferase reporter by the dATF-2 mutants. (Top) The structures of dATF-2 that were used are indicated. The results of the *trans*-activation assays shown below are summarized on the right. (Bottom) S2 cells were transfected with the CRE-containing luciferase reporter together with a plasmid expressing various forms of ATF-2. Luciferase assays were then performed. The averages and standard deviations of three experiments are indicated.

further confirm that the dMKK3–dp38 pathway induces the phosphorylation of dATF-2, we investigated the effect of dp38 and dMKK3 on the *in vivo* phosphorylation of dATF-2 (Figure 3G). Overexpression of dp38b with FLAG-dATF-2 slightly enhanced dATF-2 phosphorylation, even in the absence of stress (Figure 3G, top). Moreover, the phosphorylation levels of dATF-2 were strongly enhanced by the co-expression of dp38a (Figure 3G, bottom). On the other hand, overexpression of dMKK3 together with the DN form of dp38b (AGF), whose dMKK3 phosphorylation sites have been mutated, did not induce the phosphorylation of dATF-2 at all (Figure 3G, top). Furthermore, we observed that overexpression of both dJNK and Hep had no effect on the phosphorylation of dATF-2 (our unpublished data). These results indicate that dATF-2 is an *in vivo* target of the dMKK3–dp38 MAPK pathway, but not the Hep–dJNK pathway.

To confirm that dATF-2 is really phosphorylated at Thr-59 and Thr-61 by dp38 *in vivo*, FLAG-tagged wild-type or T59/61A mutant of dATF-2 was expressed in S2 cells and labeled with [³²P]orthophosphate (Figure 3H). Treatment of transfected cells with sorbitol enhanced the degree of phosphorylation of wild-type dATF-2, but not that of T59/61A

mutant. Furthermore, the sorbitol-induced phosphorylation of dATF-2 was blocked by the p38-specific inhibitor SB203580. Thus, dp38 seems to phosphorylate dATF-2 *in vivo* at Thr-59 and Thr-61.

Transcriptional Activation by dATF-2

To examine whether, like mammalian ATF-2, the N-terminal region of dATF-2, which contains the dp38b phosphorylation sites, can act as a transcriptional activation domain, various forms of dATF-2 were fused to the DNA binding domain (DBD) of GAL4 and their *trans*-activating capacity was investigated (Figure 4A). Thus, S2 cells were transfected with the GAL4 site-containing reporter together with the plasmid that expresses one of the GAL4-dATF-2 fusion proteins, and the luciferase activity was measured (Figure 4A). The GAL4-fusions containing the full-length dATF-2 and the N-terminal 274 amino acids had similar *trans*-activating capacities, whereas the GAL4-dATF-2 fusions containing the N-terminal 150 amino acids had a stronger activity. Consistent with this was that the GAL4-dATF-2 fusions lacking the N-terminal 150 amino acids of dATF-2 did not enhance luciferase expression at all. These results indicate that the N-terminal 150 amino acid region of dATF-2 acts as the

Figure 5. Enhancement of dATF-2-dependent *trans*-activation by dp38/dMKK3. (A) dp38/dMKK3 stimulate the CRE-mediated *trans*-activation of dATF-2. S2 cells were transfected with the CRE-containing luciferase reporter together with the plasmids that express the proteins indicated below and luciferase assays were performed. The averages and standard deviations of three experiments are indicated. (B) Stimulation of GAL4-dATF-2-dependent *trans*-activation by dp38b/dMKK3. S2 cells were transfected with the GAL4 site-containing luciferase reporter together with the plasmids that express the proteins shown below and luciferase assays were performed. (C) Inhibition of dATF-2-induced *trans*-activation by the p38 inhibitor. SL2 cells were transfected with the CRE-containing reporter and the dATF-2 expression plasmid or the control plasmid and treated with increasing concentrations of SB203580. Luciferase assays were then performed.

transcriptional activation domain and also that the C-terminal half of dATF-2 has an inhibitory effect on N-terminal region-dependent transactivation. This also has been observed with mammalian ATF-2 (Li and Green, 1996). Furthermore, alanine substitution of the Thr-59 and Thr-61 residues in the GAL4-dATF-2 fusions containing full-length dATF-2 or the N-terminal 150 amino acids dramatically decreased their *trans*-activating capacities. Thus, the N-terminal region of dATF-2 acts as a transcriptional activation domain in response to its dp38-induced phosphorylation.

We then examined the capacity of various forms of dATF-2 to *trans*-activate the CRE-containing luciferase reporter (Figure 4B). Wild-type dATF-2 stimulated the luciferase expression from this promoter, which indicates that dATF-2 stimulates transcription by directly binding to the CRE. The alanine mutations of Thr-59 and Thr-61 dramatically decreased this *trans*-activating capacity of dATF-2. Moreover, when the basic region was deleted (DBR) or the leucine zipper structure was mutated (L34P), the *trans*-acti-

vating capacity of the protein was almost completely abrogated. This is contrary to what was observed when the same mutations were introduced into GAL4-dATF-2, because these mutations had no effect on the *trans*-activating capacity of the protein (Figure 4A). This is because the binding to DNA by GAL4-dATF-2 was mediated by the GAL4 DBD. These results show that dATF-2 binds to CRE via the b-ZIP domain and that the Thr-59 and Thr-61 residues are required for the transactivating activity of dATF-2.

Regulation of dATF-2 Activity by the dMKK3-dp38 Pathway

We next examined the effect of dATF-2 phosphorylation on its *trans*-activating capacity (Figure 5A). When dp38b was coexpressed with dATF-2 and the CRE-containing luciferase reporter in S2 cells, the dATF-2-dependent stimulation of luciferase expression was enhanced approximately twofold. Coexpression of both dp38b and dMKK3 further enhanced

the *trans*-activating capacity of dATF-2 in a dose-dependent manner, which indicates that the dMKK3–dp38b pathway enhances dATF-2 activity. Similar results were observed with p38a. In contrast, coexpression of dJNK and/or Hep did not stimulate dATF-2 activity.

To further confirm that the *trans*-activating capacity of dATF-2 is enhanced by the dMKK3–dp38 pathway, we examined the transcriptional activation of GAL4–dATF-2 (Figure 5B). GAL4–dATF-2 stimulated the luciferase expression from the GAL4 site-containing luciferase reporter, and this activity was enhanced by coexpression of dMKK3 and dp38b in a dose-dependent manner. However, coexpression of Hep and dJNK did not enhance GAL4–dATF-2–dependent transcriptional activation. In contrast, the *trans*-activating capacity of GAL4–dJun was enhanced by the coexpression of Hep and dJNK, but not by the coexpression of dMKK3 and dp38b. We also investigated the effect of SB203580 on the *trans*-activating capacity of dATF-2 and found that the dATF-2-mediated transcriptional activation from the CRE-containing promoter was inhibited by SB203580 in a dose-dependent manner (Figure 5C). In addition, the dp38b/dMKK3-enhanced dATF-2 activity was also almost completely inhibited by SB203580. These results support the notion that dATF-2-dependent transcriptional activation is enhanced by the dMKK3–dp38 pathway but not by the Hep/dJNK pathway.

Expression of dATF-2 in *Drosophila*

To examine the pattern of dATF-2 expression during embryogenesis, whole-mount embryos were hybridized with an antisense RNA probe synthesized from a dATF-2 cDNA template. dp38a and dp38b expression also was examined. We found that the dATF-2 gene is expressed throughout embryonic development (Figure 6). A high level of maternal deposition similar to that observed for dMKK3 and dp38 (Han *et al.*, 1998b) was observed. In the later stages, zygotic expression is present in most tissues. The expression of dATF-2, dp38a, and dp38b is particularly evident in the leading edge of the cells at stage 12 (Figure 6, arrows).

dATF-2 Does Not Function Downstream of dJNK in Dorsal Closure

To confirm that dATF-2 is not activated by JNK *in vivo*, we investigated whether dATF-2 is involved in the process of dorsal closure during embryonic development, which is a process in which dJNK (Bsk) and dJun play a critical role. The dorsal closure occurs during mid-embryogenesis and involves cell shape changes but not cell division. The cells of the leading edge of the ventrolateral epidermis elongate and stretch in the dorsoventral axis followed by rows of epidermal cells stretch over the surface of amnioserosa cells. As shown in Figure 6, dATF-2 and dp38a and dp38b expression is evident in the leading edge of cells at stage 12 (Figure 6, arrows), raising the possibility that dATF-2 plays some role in the process of dorsal closure. To test this, we injected dATF-2 dsRNA into embryos. Embryonic lethality was not observed in 83% of the injected embryos ($n = 153$). Moreover, the 17% of embryos that showed a lethal phenotype did not clearly reveal the dorsal-open phenotype (Figure 7A, d). Most of the embryos injected with dp38 ds-RNA also did not exhibit the embryonic lethality (our unpublished data). In contrast, injection of dJun ds-RNA caused the dorsal-closure defects in 79.4% of the injected embryos ($n = 107$) (Figure 7A, c) that are commonly observed in the Hep, Bsk, dJun, and dFos mutants (Figure 7A, b) (Glise *et al.*, 1995; Riesgo-Escovar *et al.*, 1996; Sluss *et al.*, 1996; Glise and Noselli, 1997; Hou *et al.*, 1997; Riesgo-Escovar and Hafén,

Figure 6. Embryonic mRNA expression patterns of dATF-2, dp38a, and dp38b. Digoxigenin-labeled cDNA probes were incubated with wild-type embryos, and the hybridized probes were visualized by an ALP reaction. Panels at stage 2, before pole cell formation and zygotic gene expression. Panels at stage 10, when the germ band has been fully extended. Panels show stage 13, when the germ band has been retracted and the midguts have fused. Arrow indicates the leading edge of cells.

1997). We also found that transgenic embryos that expressed DN-dATF-2 did not exhibit the dorsal-open phenotype (our unpublished data). We also used a reporter gene assay with SL2 cells to test whether dATF-2 can activate the transcription of the *dpp* gene, which is one of the dJNK–dJun pathway-regulated target genes participating in the dorsal closure. However, reporter activation by dATF-2 was not observed (our unpublished data). Thus, dATF-2 is not activated by JNK, at least not during the process of dorsal closure.

Genetic Interaction between ATF-2 and dp38

To examine whether dATF-2 functions in the dp38 signaling pathway *in vivo*, we tested the genetic interaction between dATF-2 and dp38 in wing pattern formation. ATF-2 forms a homodimer and a heterodimer with Jun, but not with CREB, CREM, Fos, and so on (Hai and Curran, 1991; Matsuda *et al.*, 1991), and we previously demonstrated that the DN form of ATF-2, which has the b-ZIP domain and lacks the N-terminal activation domain, blocks the function of ATF-2, mainly by squelching normal ATF-2 by forming a dimer via a leucine zipper, not by competing for binding to CRE (Sano *et al.*, 1999). Therefore, DN-dATF-2 is likely to mainly block the

Figure 7. dATF-2 acts in the dp38 pathway, but not in the dJNK pathway. (A) dATF-2 is not involved in the dJNK-dependent dorsal closure of embryos. Lateral view of the cuticle phenotype of mock-injected (a), dJun dsRNA-injected (c), dATF-2 dsRNA-injected (d), and *bsk* mutant (b) embryos as a control for comparison. The wild-type cuticle illustrates the regular spacing of the denticle belt on the ventral side and the complete closure of the epidermis on the dorsal side (a). The *bsk* homozygous mutant embryos show a dorsal-open phenotype that is represented by the lack of the thoracic and abdominal dorsal cuticle (b). (c) Injection of dJun dsRNA into the stage 2–4 embryos (precellular blastoderm) caused the dorsal closure defect in 79.4% of the embryos ($n = 107$). (d) Embryos ($n = 153$) (83%) injected with dATF-2 dsRNA did not show a lethal phenotype. Moreover, the 17% of the embryos that did die did not clearly reveal the dorsal-open phenotype, but they exhibited the defects of head structure (arrow) and ventral cuticles (blanket) that have never been seen in dJun dsRNA-injected embryos and the *bsk* mutant embryos. (B) Effect of altering dp38b function on the wing phenotype caused by DN-dATF-2. The wing phenotype caused by DN-dATF-2, which encodes dATF-2 lacking the N-terminal activation domain, was enhanced by driving the transgenic expression of DN-dp38b by using MS1096-GAL4. (a) Expression of green fluorescent protein in the wing disk by using the MS1096-

function of dATF-2, not other CREB/ATF. When DN-dATF-2, which contains only the C-terminal b-ZIP domain, was transgenically expressed in flies using the MS1096-GAL4 driver, which directs expression in the whole region of the wing disk (Figure 7B, a), the wing venation was severely distorted and extensive production of fragments of vein material was observed (Figure 7B, c). It was previously reported that the expression of DN-dp38b at high levels, which was generated by alanine substitution of the Thr-183 MAPKK target site, leads to the weak abnormal pattern formation in certain fraction of the adult flies, including the generation of ectopic vein fragments and a reduction in the distance between the veins (Adachi-Yamada *et al.*, 1999). However, as reported previously (Adachi-Yamada *et al.*, 1999), when DN-dp38b was expressed at low levels, an aberrant wing phenotype was not induced (Figure 7B, d). We found, however, that when the DN forms of both dATF-2 and dp38b were coexpressed, significantly more severe wing formation defects were observed (Figure 7B, e). This supports the notion that dATF-2 functions in the dp38b signaling pathway. Furthermore, when *Drosophila* MEKK1 (dMEKK1), a MAPKK kinase that also acts in the p38 MAPK pathway, was expressed in the wing disk using the MS1096-GAL4 driver, severe defects in wing formation were also observed (Figure 7B, f). These defects were partly suppressed by the coexpression of DN-dATF-2 (Figure 7B, g), which supports the notion that dATF-2 acts in the dMEKK1–dp38b pathway.

Expression of DN-dATF-2 by Embryos Leads to Hypersensitivity to Osmotic Stress

The *Drosophila* MEKK1–dp38 pathway is known to be involved in the response to environmental stresses such as increased osmolarity (Inoue *et al.*, 2001). To determine whether dATF-2 plays a role together with dp38 in the response to stress, we examined whether the expression of DN-dATF-2 affects the sensitivity of embryos to osmotic stress (Table 1). The expression of DN-dATF-2 was induced by heat shock promoter-GAL4. The embryos showed normal viability when bred into normal culture medium. However, when the culture medium contained 0.2 M NaCl, their viability decreased to ~40%. In addition, expression of both DN-dATF-2 and DN-dp38b further decreased the viability to 24%. Furthermore, coexpression of dMEKK1a together with DN-dATF-2 partly suppressed the effect of DN-dATF-2. Thus, dATF-2 functions together with dp38b and dMEKK1a in the response to osmotic stress.

dATF-2 Is a Major Regulator of Stress-inducible Gene Expression

To investigate whether dATF-2 is needed for stress-induced gene expression, we determined by DNA array analysis how many and which stress-induced genes are regulated by dATF-2. Thus, RNAs prepared from S2 cells treated with or without 0.4 M sorbitol were first subjected to DNA array analysis with the Affymetrix *Drosophila* genome array, which contains >13,500 transcripts. The results indicated that osmotic stress increased the expression of 107 genes more than twofold. S2 cells were then transfected with dATF-2 dsRNA to

GAL4/UAS driver. (b–g) Adult wing phenotypes. (b) Wild-type fly. (c–e) Flies carrying one copy of UAS-DN-dATF-2 (c) or UAS-DN-dp38b (d) or both (e). DN-dp38b is the dp38b mutant whose MAPKK target residue Thr-183 has been replaced with Ala. (f and g) Flies carrying one copy of UAS-dMEKK1 (f) or both UAS-dMEKK1 and UAS-DN-dATF-2 (g). DN-dATF-2 suppresses the dMEKK1 wing phenotype.

Table 1. Sensitivity to osmotic stress by loss of dATF-2 activity

Genotype ^a	Viability (%)		
	NaCl ^b +		NaCl –
	HS –	HS ^c +	HS ^c +
<i>UAS-dATF-2^{DN}</i>	94 (206/219)	41 (100/243)	96 (235/245)
<i>UAS-D-p38b^{DN}</i>	95 (224/236)	96 (266/277)	93 (200/215)
<i>UAS-dATF-2^{DN}, UAS-D-p38b^{DN}</i>	90 (187/208)	24 (49/203)	92 (208/226)
<i>UAS-D-MEKK1a</i>	95 (200/211)	90 (169/188)	93 (183/197)
<i>UAS-dATF-2^{DN}, UAS-D-MEKK1a</i>	96 (176/183)	67 (151/225)	92 (192/209)
<i>hs-GAL4</i>	98 (318/324)	94 (309/331)	99 (302/305)
<i>D-MEKK1^{Utr36}</i>	13 (21/163)		

Viability was calculated from the number of adult flies per the number of first larvae derived from mating *hs-Gal4* females with UAS transgenic males.

^a Transgenic flies shown in genotype contain one copy each of *hs-GAL4* and UAS transgene except for *D-MEKK1* mutant.

^b A final concentration of 0.2 M NaCl was added to the culture medium at 25°C.

^c Heat shock treatment (HS) was carried out at 30°C for 15 min per day during larval period.

down-regulate dATF-2 and treated with sorbitol. RNAs from these cells were then used for array analysis. We confirmed that dATF-2 dsRNA transfection markedly decreased the endogenous dATF-2 protein levels (Figure 1E). The array analysis indicated that introduction of dATF-2 dsRNA decreased the expression levels of 233 genes by more than twofold. Comparison of the 107 genes that were induced by the high osmolarity with the 233 genes that were down-regulated by dATF-2 dsRNA indicated that 43 genes are common (Figure 8A). This means that ~40% of the genes that were induced by osmotic stress are regulated by dATF-2, which indicates that dATF-2 is a major inducer of gene expression upon osmotic stress. These 43 genes include seven immune system genes, six genes encoding cell surface or cuticle proteins, five genes encoding transporters, five genes encoding endopeptidases, and two genes encoding receptors (Figure 8B).

DISCUSSION

In the present study, we identified and characterized dATF-2. The amino acid sequences of the b-Zip domain and the region containing the p38/JNK phosphorylation sites of mammalian ATF-2 are well conserved in dATF-2. However, dATF-2 lacks the N-terminal zinc finger domain that is conserved in the three members of the mammalian ATF-2 family (ATF-2, CRE-BPa, and ATF-a). The N-terminal zinc finger motif and the adjacent region that contains the p38/JNK phosphorylation sites in the mammalian ATF-2 together act as the transcriptional activation domain (Matsuda *et al.*, 1991). Therefore, the mediators that regulate the transcriptional activation of mammalian ATF-2 and dATF-2 may have different characteristics.

We found that extracellular stress such as UV or osmotic stress induces the dp38-induced phosphorylation of dATF-2 at Thr-59 and Thr-61 and that this increases the *trans*-activation capacity of dATF-2. Although mammalian ATF-2 is well known to be phosphorylated not only by p38 but also by JNK, we found that dJNK neither directly phosphorylated dATF-2 nor enhanced dATF-2-dependent transcription. Furthermore, transgenic embryos expressing DN-dATF-2 or dATF-2 dsRNA did not clearly reveal the dorsal-open phenotype that is common to the Hep, Bsk, dJun, and dFos mutants (Glise *et al.*, 1995; Riesgo-Escovar *et al.*, 1996;

Sluss *et al.*, 1996; Hou *et al.*, 1997; Riesgo-Escovar and Hafen, 1997). The entire amino acid sequence of JNK1 shares 65% identity with dJNK (Riesgo-Escovar *et al.*, 1996; Sluss *et al.*, 1996), and the ~50-amino acid stretch within the N-terminal domain of mammalian ATF-2 that contains the phosphorylation sites is also well conserved in dATF-2 (59% identity). Therefore, it is surprising that dJNK cannot phosphorylate dATF-2, unlike what is observed for mammalian JNK and ATF-2. Furthermore, we found that although dATF-2 is phosphorylated only by dp38, dJun is phosphorylated by both dp38 and dJun (Figure 3A). In contrast, mammalian ATF-2 is phosphorylated by both p38 and JNK, whereas Jun is phosphorylated only by JNK (Han *et al.*, 1998b). It is worth noting that ATF-a is not phosphorylated by JNK (De Graeve *et al.*, 1999). This may raise the possibility that a regulation mechanism of dATF-2 resembles to that of ATF-a, and that an ancestral ATF-2/CRE-BPa gene were derived from a duplicated ATF-a-like gene. The relationship between SAPKs and transcription factors in *Drosophila* and mammals may be useful in understanding how the stress-inducible gene expression system is established during evolution.

The GAL4-dATF-2 fusions containing the N-terminal 150 amino acids had a stronger activity than those containing the N-terminal 274 amino acids (Figure 4A), indicating that the region between amino acids 150 and 274 has a negative effect on the activation domain of dATF-2. In the case of vertebrate ATF-2, the b-ZIP DBD suppresses the ATF-2 activation domain via intramolecular interaction (Li and Green, 1996). This difference may suggest that the mechanism by which the C-terminal region suppresses the activation domain is different between vertebrate ATF-2 and dATF-2. It is interesting whether the region between amino acids 150 and 274 of dATF-2 affects the stability or conformation of dATF-2 protein. Wild-type dATF-2 stimulated the luciferase expression from the CRE-containing promoter under nonstimulated condition (Figure 4B). Because the alanine mutants of Thr-59 and Thr-61 dramatically decreased this *trans*-activating capacity of dATF-2, phosphorylation of these residues seems to be essential for *trans*-activating capacity of dATF-2. These results suggest the possibility that the Thr-59 and Thr-61 residues are phosphorylated at low levels even under nonstimulated condition. This could be due to the low levels of TNF- α or IL-1 involved in serum. Alternatively, other

Figure 8. dATF-2 is a major regulator of osmotic stress-induced gene expression. (A) Comparison of the high osmolarity-induced genes with the dATF-2-regulated genes. RNAs were prepared from S2 cells treated with 0.4 M sorbitol or control solvent. RNAs also were prepared from sorbitol-treated S2 cells that had been transfected with dATF-2 dsRNA. The cDNA probes prepared from these RNAs were then used for DNA array analysis. The expression of 107 genes was induced more than twofold by sorbitol, whereas the expression of 233 genes was reduced by dATF-2 dsRNA. These genes were identified and comparison between the two groups indicates that 43 genes are common. (B) Functional classification of those of the 43 genes to which a molecular function could be assigned.

Using two different assay systems, we have demonstrated at the genetic level that dATF-2 acts in the dp38 signaling pathway. First, we showed that expression of DN-dp38b enhanced the aberrant wing phenotype caused by DN-dATF-2. It has been reported previously that dp38b acts downstream of the Dpp receptor Tkv, because DN-dp38b expressed in the wing imaginal disc caused a phenotype resemble to the mutant of *dpp* (*decapentaplegic*) that is a *Drosophila* homologue of mammalian bone morphogenetic protein/TGF- β /activin superfamily (Adachi-Yamada *et al.*, 1999). Therefore, dATF-2 may functions in the Dpp signaling pathway. This may be consistent with our previous finding that mammalian ATF-2 is phosphorylated by TGF- β signaling via TAK1 and p38, and it then directly binds to the Smad3/4 complex to synergistically activate transcription with Smad3/4 (Sano *et al.*, 1999). We also demonstrated that DN-dp38b coexpression enhanced the sensitivity of embryos expressing DN-dATF-2 to high osmolarity. Thus, dATF-2 acts in the dp38 signaling pathway, at least in wing pattern formation and the response to osmotic stress. However, no oocyte defects were observed in the transgenic flies expressing DN-dATF-2, although the dp38 MAPK pathway is known to be required during oogenesis for asymmetric egg development (Suzanne *et al.*, 1999). Thus, dATF-2 may function only in some specific events that are regulated by the dp38 signaling pathway.

Our DNA array analysis indicated that ~40% of the genes that are induced by osmotic stress are also regulated by dATF-2, which indicates that dATF-2 is a major inducer of osmotic stress-inducible gene expression. These genes encode cell surface and cuticle proteins, transporters, and receptors, and various endopeptidases (Figure 8B). It is not surprising that osmotic stress may increase the production of cell surface proteins, including some receptors. In addition, the endopeptidases may be produced because high osmolarity may increase the denaturation of proteins, which must then be degraded by the cell. The dATF-2 target genes also include seven immune response genes, namely, several encoding antimicrobial peptides and one encoding a peptidoglycan recognition protein, which binds to the peptidoglycans of bacterial cell walls and triggers immune responses (Hoffmann, 2003). It has been reported that LPS increases the kinase activity of dp38 (Han *et al.*, 1998b). Consequently, dp38-phosphorylated dATF-2 may directly induce these immune response-related genes. However, it also has been shown that overexpression of dp38 inhibits the expression of immune response genes (Han *et al.*, 1998b). This could be explained by the possibility that dp38 overexpression may inhibit the p38 signaling pathway by activating negative feedback regulatory mechanisms, such as the p38 α -induced decrease of MKK6 mRNA stability in mammalian cells (Ambrosino *et al.*, 2003). In *Drosophila*, Gram positive bacteria and fungi predominantly induce the Toll signaling pathway to activate genes such as Drosomycin, whereas Gram negative bacteria activate the Imd pathway to activate genes such as Diptericin (Hoffmann, 2003). DNA array analysis indicated that both Drosomycin and Diptericin are regulated by dATF-2, which suggests that dATF-2 may be a component of both the Toll and Imd pathways. Further analyses of dATF-2 will most likely enhance our understanding of the molecular mechanisms involved in the *Drosophila* immune system.

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kinase(s) also may phosphorylate these residues, because vertebrate ATF-2 is activated by Raf-MEK-ERK pathway via phosphorylation of Thr-71 (Ouwens *et al.*, 2002).

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