# The AP-1 repressor, JDP2, is a bona fide substrate for the c-Jun N-terminal kinase

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**Abstract The Jun dimerization protein 2 (JDP2) is a novel member of the basic leucine zipper family of transcription factors. JDP2 binds DNA as a homodimer and heterodimer with ATF2 and Jun proteins but not with c-Fos proteins. JDP2 overexpression represses activating protein 1 transcription activity. Whereas JDP2 mRNA and protein levels are stable following different cell stimuli, JDP2 is rapidly phosphorylated upon UV irradiation, oxidative stress and low levels of translation inhibitor. The c-Jun N-terminal kinase phosphorylates JDP2 both in vitro and in vivo. JDP2 contains a putative consensus JNK docking-site and a corresponding phosphoacceptor site. Substitution of threonine 148 to an alanine residue blocks JNK-dependent JDP2 phosphorylation. Our data indicate that JDP2 is a bona fide substrate for the c-Jun N-terminal kinase. The precise role of JDP2 phosphorylation on its function is not yet known.** 

*Key words:* c-Jun N-terminal kinase; Protein phosphorylation; Phosphoacceptor; UV irradiation; Activating protein 1; Jun dimerization protein 2

## 1. Introduction

The activating protein 1 (AP-1) is an immediate early transcription factor composed of multiple protein families such as the Jun, Fos and ATF [1,2]. AP-1 is an important transcription factor in multiple cellular processes as diverse as cell proliferation, differentiation and apoptosis [1].

All AP-1 proteins contain a basic region responsible for DNA binding followed by a leucine zipper motif (bZIP) important for homo- and heterodimerization between family members. The Jun family is able to homodimerize as well as heterodimerize with Fos and ATF family members [3,4]. Heterodimerization serves to increase their affinity towards the TPA response element (TRE) and modify their DNA binding specificity. The AP-1 components are subjected to multiple levels of regulatory mechanisms and modifications that result in dramatic changes in their transcription activity. In addition to subunit exchange, the transcription of various AP-1 components is regulated upon serum and growth factor stimulation. More importantly, post-transcriptional modifications play a major role in the regulation of AP-1 activity [1,2]. The AP-1 components are subjected to phosphorylation by multiple kinases such as the c-Jun N-terminal kinase (JNK) [5,6] and the Fos regulating kinase (FRK), yet to be identified [7]. The phosphorylation occurs at their transcription activation domain, which strongly potentiates their transcriptional activity, presumably by facilitating interaction with CBP/p300 co-activators [8] and/or resulting in prolonged half-life [9,10]. In addition, casein kinase II phosphorylation of c-Jun inhibits its DNA binding activity [11]. Recently, we have isolated a novel member of the bZIP protein family designated: JDP2 [12]. JDP2 was isolated based on its ability to interact with c-Jun bZIP domain. JDP2 contains a leucine zipper domain that is most closely related to ATF3 [13] exhibiting 60% amino acid homology. JDP2 is able to homodimerize and heterodimerize with both Jun family members and ATF2 proteins [14] but not with c-Fos proteins. JDP2 is able to bind both TRE and CRE DNA binding elements, either as a homodimer or heterodimer [12,14]. Unlike Fos proteins, JDP2 protein levels remain unchanged following growth factor and serum stimulation. JDP2 overexpression in fibroblast cells results in strong inhibition of AP-1 transcriptional activity. Recently, we have showed that JDP2 can inhibit p53 induction as a result of UV irradiation, resulting in inhibition of p53-mediated apoptosis [15]. Since JDP2 has the potential to counteract AP-1 activity and the fact that AP-1 plays a central role in signal transduction, we studied the regulation of JDP2 following different cell stimuli. In this paper, we describe the process whereby we identified that JDP2 undergoes rapid phosphorylation upon different stress conditions. We identified the JNK as the kinase responsible for JDP2 phosphorylation and mapped threonine 148 as the phosphoacceptor site.

## 2. Materials and methods

### 2.1. Cell culture

*2.1.1. Cell culture and stimulation.* Mouse fibroblast cells (NIH 3T3), Rat intestinal cell lines IC18 and RI [16] and Rat1A cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/ml D-glucose 10% fetal calf serum in a 5% $CO_2$ incubator.

*2.1.2. Transfection.* NIH 3T3 cells were transfected using lipofectamine (Gibco BRL Ltd.) with 6 μg of DNA and 15 μl of lipofectamine according to the manufacturer's instructions.

### 2.2. Cell stimulation

UV irradiation was applied using a 312 nm table lamp. The medium was removed prior to 2 min UV irradiation. Typically the cells were harvested 30 min following UV irradiation unless otherwise indicated. Serum-starved cells grown in 1% serum for 24 h, were treated with: anisomycin (50 ng/ml), $H_2O_2$ (5 mM), tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA, 50 ng/ml), for 30 min prior to nuclear extract preparation [17].

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*2.3. Potato acid phosphatase treatment*

Nuclear cell extract (50 μg) was incubated for 30 min at 37°C with 40 μg of potato acid phosphatase (Cat# 108227 Roche Inc.) in 10 mM PIPES–HCl (pH 6.0) buffer.

*2.4. Kinase induction*

SRα expression plasmid encoding HA-JNK was transfected into NIH 3T3 cells. 30 min before harvesting, cells were induced by UV irradiation. Whole cell extract was prepared according to [5] and immunoprecipitation, and an in vitro kinase assay was performed with anti-HA antibodies (Babco Inc.) as described in [18]. In-gel kinase assay was performed as described in [5].

*2.5. Protein purification*

GST and His fusion proteins were purified with glutathione (Sigma Ltd.) or nickel (Qiagen Inc.) agarose beads, respectively according to the manufacturer's instructions. In principle, following elution with 5 mM glutathione and 100 mM EDTA, respectively, purified proteins were dialyzed against PBS containing 10% glycerol. PGEX-kg-TAT plasmid was a kind gift from Dr. Michael Olson. TAT-JDP2 proteins were extracted and purified as described [19]. TAT-JDP2 was cleaved by overnight digestion with thrombin (Sigma Cat# T7513) at 4°C followed by incubation with *p*-aminobenzamidine beads (Sigma A-7155) for 1 h to remove the thrombin.

*2.6. Protein detection*

Immunoprecipitation and Western blot analysis were performed with anti-HA monoclonal (12CA5) antibodies (Babco Inc.), anti-c-Myc 9E11 monoclonal antibodies and anti-JDP2 antibodies [12]. All incubations and washing steps were performed using 10% low fat milk in phosphate buffer saline except for the incubation with anti-JDP2 antibodies where the buffer was supplemented with NaCl to a final concentration of 300 mM and 0.1% Tween 20.

*2.7. Plasmids*

Mammalian His-JDP2 was expressed using the pCDNA 3.1 expression plasmid (Invitrogen Inc.); SRα expression vectors were previously described [18,20]. pET-His expression plasmids were based on pET15b bacteria expression vector (Novagen Inc.).

Site-directed mutagenesis was performed using the Chameleon kit (Stratagene Inc.) according to the manufacturer's instructions and using the appropriate oligonucleotides. The mutations were confirmed by DNA sequencing. Bacterial histidine-tagged plasmids encoding JDP2 deletion mutants were constructed by polymerase chain reaction (PCR) approach with appropriate primers to result in frame fusion of JDP2 with the pcDNA histidine-tag. DNA sequencing was performed to verify that JDP2 is free from PCR mutations.

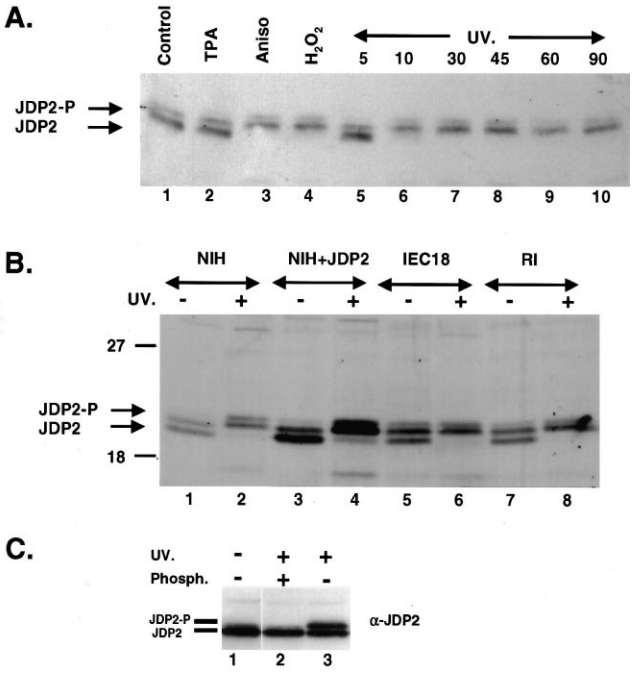


Fig. 1. Stress stimuli induce changes in JDP2 migration. A: Serum-starved NIH 3T3 cells were treated as indicated: TPA (50 ng/ml), anisomycin (50 ng/ml), 5 mM $H_2O_2$ and UV irradiation (312 nm for 2 min). Nuclear extract was prepared 30 min (otherwise as indicated) following cell stimulation. Extract was separated by 16 cm long 12.5% SDS–PAGE immobilized to Hybond (Amersham Inc.) nitrocellulose membrane. Western blot analysis was performed using anti-JDP2 as primary antibody and HRP-conjugated-protein-A followed by chemiluminescent reaction and autoradiography. The migration of JDP2 is indicated. B: The indicated cell lines were exposed to UV irradiation 30 min prior to cell harvesting. NIH 3T3 stably transfected with JDP2 (NIH+JDP2) [15], IEC18 and RI [16]. Cell extract and Western blot analysis was performed as described in A. C: Rat1A cells stably transfected with JDP2 wild type were serum-starved (24 h, 1% serum) and UV-irradiated where indicated (UV+). Nuclear extract (50 μg) was treated with potato acid phosphatase (Phosph.+). All protein extracts were incubated at 37°C for 30 min followed by separation by 12% SDS–PAGE and Western blot analysis with anti-JDP2 antibodies.

## 3. Results

Previous studies have shown that JDP2 protein levels are stable following serum stimulation [12]. To examine the expression levels of JDP2 protein following different cell stimuli, we exposed NIH 3T3 cells to phorbol ester treatments (TPA), low levels of translational inhibitor anisomycin, oxidative stress ($H_2O_2$) and UV irradiation 30 min prior to cell extraction (unless otherwise indicated). Nuclear cell extract prepared from treated cells was separated by a long 12.5% SDS–PAGE followed by Western blotting and incubation with anti-JDP2 antibodies. JDP2 typically migrates as a doublet band at a size of 20–21 kDa (Fig. 1A). These bands may correspond to an alternative ATG usage at the 5′-end of the transcript. Expression of JDP2 fused to an upstream ATG consensus sequence appears as a single band (see for example Fig. 3D). JDP2 expression levels are unchanged following cellular treatments by various stimuli. Following anisomycin, $H_2O_2$ treatments or UV irradiation, slower migration of JDP2 cross-reactive band was observed (Fig. 1A). Similar aberrant migration occurs in all cell lines tested of mouse and rat origin (Fig. 1B) and human origin (not shown). No change in migration was observed upon TPA treatments and 5 min following UV irradiation. In addition, nuclear extract prepared 24 h after UV irradiation still showed the upshift in JDP2 migration (not shown). To test the possibility that JDP2 aberrant migration is due to phosphorylation, cell extract derived from UV-irradiated cells was treated with potato acid phosphatase and was subjected to Western blot analysis using anti-JDP2 antibodies (Fig. 1C). The migration of JDP2 protein following phosphatase treatment was identical to the corresponding protein derived from unstimulated cells. These results suggest that JDP2 is phosphorylated upon different stress stimuli that are known to activate the stress activating protein kinases, SAPK [21,22]. To test whether or not the JNK is able to phosphorylate JDP2, we performed an in-gel kinase assay with either bacterially purified GST-c-Jun or bacterially purified histidine-tagged JDP2 proteins. In this assay the purified proteins are polymerized within the separating gel. Whole cell extracts derived from: untreated cells, TPA-induced, UV-irradiated and anisomycin-treated cells were resolved by SDS–PAGE. Following electrophoresis, the gel was subjected to a denaturation with 6 M urea followed by a stepwise renaturation protocol. Subsequently, the gel was incubated with kinase buffer in the

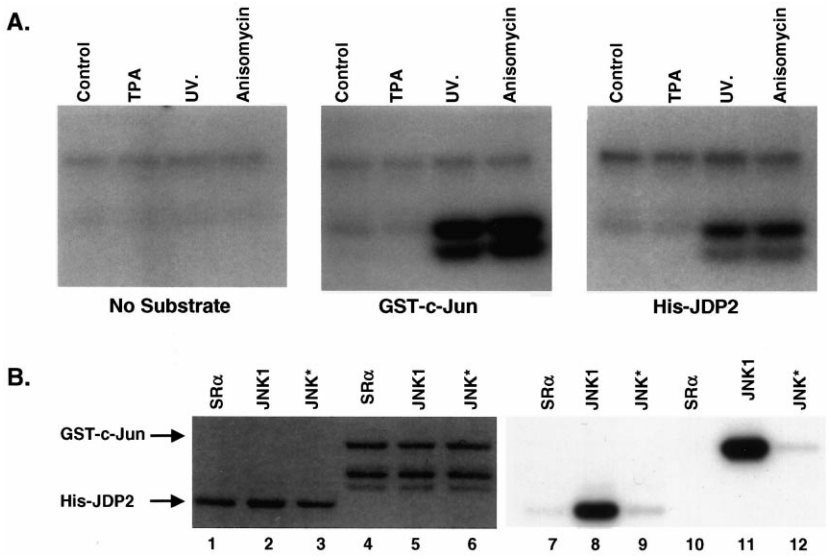


Fig. 2. In vitro phosphorylation of bacterially purified JDP2 by the stress activated protein kinase, JNK1. A: Cell extracts derived from NIH 3T3 cells treated as indicated were separated by 10% SDS–PAGE. The separating gels were polymerized in the absence (no substrate, left panel) or in the presence of either bacterially purified (40 μg/ml) GST-c-Jun (GST-c-Jun, middle panel) or bacterially purified His-JDP2 (right panel). After electrophoresis, the gels were treated as described in [5], dried and exposed to autoradiography for 12 h. B: Bacterially purified His-JDP2 was used as a substrate in an in vitro kinase assay using immunopurified activated HA-JNK1 protein. Coomassie brilliant blue staining (left panel) and [$\gamma$-$^{32}$P]ATP incorporation is shown (right panel). Cell extracts corresponding to either vector transfected (SR$\alpha$) or the indicated HA-tagged kinases were used in an in vitro kinase assay using either bacterially purified His-tagged JDP2 (His-JDP2) or bacterially purified GST-c-Jun. The immunopurified protein corresponding to the inactive kinase HA-JNK1-APF mutant [6] (JNK*) was used in the kinase assay (lanes 9 and 12).

presence of [$\gamma^{32}$P]ATP, dried and exposed to autoradiography (Fig. 2A). The gels containing either GST-c-Jun (Fig. 2A, mid panel) or His-JDP2 (Fig. 2A, right panel) exhibited a significant incorporation of radiolabelled phosphate in cell extract derived from UV and anisomycin-treated cells but not from extract-derived TPA-treated cells or non-treated cells. This result suggests the presence of active kinases at position 46 and 55 kDa that are able to phosphorylate both c-Jun and JDP2. The protein kinases that are consistent with this size and activation signals may correspond to JNK1 and JNK2, respectively. The 55 kDa kinase, JNK2, exhibits a stronger signal with both GST-c-Jun and His-JDP2 which may reflect higher binding affinity of this kinase to c-Jun [18] and may suggest similar preference towards JDP2. The upper band migrating at 70 kDa that appears in both gels may correspond to an autophosphorylation of a constitutive kinase and is readily observed in the gel where no substrate was added as well (Fig. 2A, left panel). To test more directly the ability of JDP2 to serve as JNK substrate, we performed an in vitro kinase assay with immunopurified activated JNK. Cells were transfected with plasmids encoding HA-JNK1 and subsequently cells were UV-irradiated 30 min prior to cell extraction. Anti-HA 12CA5 antibody was used to immunoprecipitate HA-JNK1 followed by extensive washes. Immunopurified HA-JNK1 was incubated with either bacterially purified His-JDP2 or GST-c-Jun in the presence of [$\gamma$-$^{32}$P]ATP. Following a kinase reaction the extract was separated by SDS–PAGE, Coomassie blue-stained, dried and exposed to autoradiography (Fig. 2B). The results shows that JNK1 is able to efficiently phosphorylate His-JDP2 at levels comparable to the phosphorylation obtained with purified GST-c-Jun. Control extracts derived from cells transfected with either an empty expression vector or kinase-inactive JNK1 [6] (HA-JNK-APF) were unable to phosphorylate either His-JDP2 or GST-c-Jun, indicating that JDP2 is a direct JNK substrate. Similar analysis with HA-ERK2 resulted in no His-JDP2 phosphorylation (not shown), suggesting that JDP2 is a bona fide substrate for JNK but not for ERK. This is consistent with the fact that TPA treatment did not induce the typical upshift that is observed following JNK activation (Fig. 1).

In order to map the JNK phosphoacceptor site within JDP2, we prepared JDP2 bacteria expression plasmids encoding histidine-tagged JDP2 deletion mutants in which either the N-terminal or the C-terminal domains were deleted (Fig. 3A). Proteins were purified by nickel agarose and used in an in vitro kinase assay with activated HA-JNK2 (Fig. 3B). Truncation of JDP2 N-terminal domain completely retained its ability to undergo phosphorylation by JNK2 (Fig. 3B). In contrast, deletion of the C-terminal domain resulted in a complete loss in JDP2 phosphorylation. This indicates that JDP2 phosphoacceptor site is located within the C-terminal domain.

The JNKs are proline-directed kinases, thus phosphorylate substrates on either a serine or a threonine residue followed by a proline residue. Within JDP2 C-terminal domain, threonine 148 is followed by a proline residue. To test whether or not threonine 148 is a JNK phosphoacceptor site, we used site-directed mutagenesis to convert threonine 148 to an alanine residue (JDP2-T148A). Bacterially purified JDP2-mutant protein harboring T148A was unable to undergo phosphorylation by activated JNK2 in vitro (Fig. 3C). In order to demonstrate that threonine 148 serves as JNK phosphoacceptor site in vivo, we established a NIH 3T3 stable cell line expressing either His-JDP2 wild type or His-JDP2-T148A mutant. Histidine-tagged JDP2 migrates significantly slower (25 kDa) as compared to the endogenous protein and therefore can be easily distinguished from the endogenous JDP2 protein. Cells were subjected to UV irradiation and nuclear extracts were separated by SDS–PAGE followed by Western blot analysis

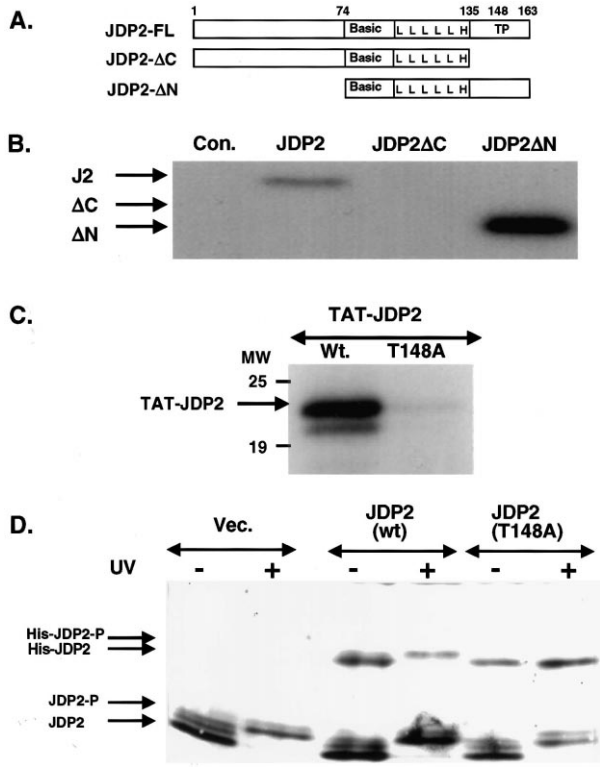


Fig. 3. Threonine 148 is JNK phosphoacceptor site in vitro and in vivo. A: Schematic diagram describing the deletion constructs used. The basic region (Basic), the leucine zipper motif (L) and the threonine-proline (TP) are indicated. The numbers on the top represent the amino acid in JDP2 in respect to the truncation performed. B: Bacterially purified His-tagged JDP2 proteins corresponding to wild type (JDP2), C-terminal truncation (JDP2ΔC) and N-terminal truncation (JDP2ΔN) were used in in vitro kinase assay as described in Fig. 2B. The autoradiograph of [$\gamma$-$^{32}$P]ATP incorporation following the kinase reaction is shown. C: Bacterially purified TAT-JDP2 wild type protein and T148A mutant protein (2 μg) were used in an in vitro kinase assay as described in Fig. 2B. The autoradiograph of [$\gamma$-$^{32}$P]ATP incorporation following the kinase reaction is shown. D: NIH 3T3 cells were stably transfected with a plasmid encoding either His-tagged JDP2 wild type (JDP2-wt) or His-JDP2-T148A. A NIH 3T3 cell line containing an empty pcDNA vector alone was used as a control (Vec.). Cells were either untreated or UV-irradiated 30 min prior to nuclear extract preparation. The extract was separated by 12.5% SDS–PAGE followed by Western blotting. JDP2 protein was detected using anti-JDP2 antibodies followed by chemiluminescent reaction and autoradiography. The migration of the endogenous JDP2 and the transfected His-JDP2 is indicated.

with anti-JDP2 antibodies (Fig. 3D). The endogenous JDP2 protein exhibited the typical slower migrating band following UV irradiation as well as cell extract derived from His-JDP2 wild type expressing cells. In contrast, no change in the migration of His-JDP2 T148A was observed following UV irradiation (Fig. 3D). This result indicates that threonine 148 serves as the JNK phosphoacceptor site in vivo.

## 4. Discussion

Although the JNK kinase has attracted the attention of many investigators, the substrates available for these enzymes are rather limited. So far, c-Jun, ATF2, Sap-1a, ELK1, c-Myc, Bcl2, p53, and NFAT4 are the best substrates characterized [22,23]. As for c-Jun [6], ATF2 [24] Sap-1a [25] and ELK1 [26], it was demonstrated that JNK phosphorylation is within their transcriptional activation domain, resulting in potentiation of their transcriptional activity. As for NFAT4, its phosphorylation was found to counteract calcineurin phosphatase action, resulting in translocation of NFAT4 to the cytoplasm and thereby inactivating NFAT4 action [27].

Two additional JNK substrates, p53 [28] and Bcl2 [29] were initially described as substrates with no obvious function. In the case of p53, it was recently demonstrated that JNK-dependent p53 phosphorylation targets p53 to ubiquitination and degradation [30], whereas, for Bcl2 it was demonstrated that JNK phosphorylation inactivates its anti-apoptotic activity [31].

In this paper, we described the identification of JDP2 as a novel substrate for the stress activating kinase JNK. Threonine 148 of JDP2 is the phosphoacceptor site and mutation of this site completely abrogated JDP2 phosphorylation both in vitro and in vivo. Endogenous JDP2 protein undergoes phosphorylation under different stress conditions such as: UV irradiation, oxidative stress and anisomycin treatments. It has been shown that the JNK phosphoacceptor site is distinct from its docking-site [23,32]. The consensus JNK docking-site found in the c-Jun delta region includes KXXX/RXXXXLXL. Indeed, such a sequence is present upstream of the JDP2 phosphoacceptor site and resides within the JDP2 leucine zipper motif. The importance of this domain for JDP2 phosphorylation is currently under investigation.

Since JDP2 function is clearly distinct from all the abovementioned substrates and serves as a transcriptional repressor, the role of JDP2 phosphorylation is clearly distinct and yet to be determined.

Using in vitro analysis (not shown), we failed to detect differences in DNA binding and association with JDP2 protein partners. One possibility could be that we used acidic residues to mimic JDP2 phosphorylation. Although acidic residue substitutions have been shown in several cases to mimic protein phosphorylation events [33–36], it may be that JDP2 phosphorylation could not, as was already demonstrated in CREB phosphorylation on serine 133, where a negative charge could not mimic phosphorylated CREB activity [37]. Moreover, since JDP2 was shown to bind to multiple DNA elements such as: CRE, TRE and CAAT sequences [12,38], one cannot exclude the possibility that binding to a specific DNA binding site, yet to be identified, is subject to differential regulation by JDP2 phosphorylation. This also may explain our failure to detect differences in JDP2 activity using reporter gene assays in which the reporter gene is placed under the control of consensus AP-1 binding sites that may not necessarily be the actual physiological JDP2 targets. With the recent advances in DNA array-based techniques one can better define the natural targets for a specific transcription factor. For example, the Wilms tumor suppressor, WT1, in transient transfection assays represses transcription from multiple DNA elements. However, using a high-density oligonucleotide microarrays technique, WT1 was demonstrated to directly activate transcription from the amphiregulin promoter [39]. WT1 activity is performed via binding to a high-affinity site within the amphiregulin promoter suggesting that WT1 may function as a transcriptional activator depending on the promoter tested.

In summary: we have shown that JDP2 serves as a bona fide substrate for the stress activating kinase, JNK. The phosphorylation occurs on threonine 148. Substitution of threo-

nine 148 to an alanine residue abrogates JDP2 phosphorylation. The conservation of this site among different species and the rapid and persistent phosphorylation may suggest that JDP2 phosphorylation may have an important functional role which is yet to be identified.

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