Differential targeting of the stress mitogen-activated protein kinases to the c-Jun dimerization protein 2

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The mitogen-activated kinases are structurally related proline-directed serine/threonine kinases that phosphorylate similar phosphoacceptor sites and yet, *in vivo*, they exhibit stringent substrate specificity. Specific targeting domains (kinase docking domains) facilitate kinase–substrate interaction and play a major role in substrate specificity determination. The c-Jun N-terminal kinase (JNK) consensus docking domain comprises of a KXXK/RXXXXLXL motif located in the δ-domain of the c-Jun N-terminal to the phosphoacceptor site. The c-Jun dimerization protein 2 is phosphorylated by JNK on Thr-148. Activating transcription factor 3 (ATF3) is a basic leucine zipper protein which is highly homologous to c-Jun dimerization protein 2 (JDP2), especially within the threonine/proline phosphoacceptor site, Thr-148. Nevertheless, ATF3 does not serve as a JNK substrate *in vitro* or *in vivo*. Using ATF3 and JDP2 protein chimaeras, we mapped the JNK-docking domain within JDP2. Although a JNK consensus putative docking site is located within the JDP2 leucine zipper motif, this domain does not function to recruit JNK to JDP2. A novel putative docking domain located C-terminally to the JDP2 phosphoacceptor site was identified. This domain, when fused to the ATF3 heterologous phosphoacceptor site, can direct its phosphorylation by JNK. In addition, although the novel JNK-docking domain was found to be necessary for p38 phosphorylation of JDP2 on Thr-148, it was not sufficient to confer JDP2 phosphorylation by the p38 kinase.

Key words: activating transcription factor 3, docking domain, phosphorylation.

INTRODUCTION

The mitogen-activated protein kinase (MAPK) signalling cascades play a pivotal role in converting extracellular signals into specific nuclear responses [1,2]. These cascades contain multiple components acting in a sequential series of phosphorylation steps whereby MAPK kinase kinases phosphorylate and activate MAPK kinases, which in turn phosphorylate the extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPKs that are considered the three best-characterized tiered cascades. The latter phosphorylate serine/threonine residues that are followed immediately by a proline residue. Although numerous proteins may contain this minimal consensus sequence, only a small number of them would serve as *bona fide* substrates. Much effort has been spent in trying to understand how substrate specificity occurs. It became apparent that MAPKs recognize their substrates by binding to specific sequences known as docking sites, typically located upstream of the substrate phosphoacceptor site [3,4]. These docking domains provide increased kinase specificity and efficiency for substrate phosphorylation. In addition, the proximity of the docking domain relative to the phosphoacceptor site serves to ensure accuracy of phosphorylation [3]. Besides the ability of a docking domain to recruit a kinase to a phosphoacceptor site located in close proximity to the same protein, it may also direct the phosphorylation of proteins lacking a docking domain but physically associated with the docking-domain-containing protein [5]. Nevertheless, a recent paper argues against this hypothesis [6]. In general, the docking sites contain a LXL motif located three–five amino acids downstream from a region containing several basic residues. The central core LXL motif clearly plays a major role in kinase targeting to transcription factors [3].

The JNK serves as a paradigm for the study of substrate–kinase interplay. The JNK-docking domain found in the δ-domain of c-Jun was defined as a JNK MAPK-docking domain. v-Jun contains identical phosphoacceptor sites to c-Jun but lacks a JNK-docking domain and thus does not serve as a JNK substrate. Similarity between the binding motifs of c-Jun, ELK1/TCF1 (ternary complex factor 1) [7] and JNK inhibitory protein (JIP)-1 [8] is found in a consensus R/KXXXXLN/EL, representing a core binding site. However, examination of the binding motifs of several JNK substrates reveals little similarity with the central core. The subtle differences between docking domains may provide preferential specificity among the multiple JNK isoforms, and also account for differences in their ability to undergo phosphorylation, depending on the signal exerted.

c-Jun dimerization protein 2 (JDP2) is a basic leucine zipper protein member of the AP-1 family [9,10]. JDP2 also interacts with activating transcription factor (ATF) 2 [10] and CCAAT/enhancer-binding protein (C/EBP) γ [11]. Unlike other members of the AP-1 family, JDP2 is expressed in all cell lines and tissues tested. JDP2 is an AP-1 repressor protein that plays a positive role in cell cycle control and was found to be important in skeletal muscle cell differentiation [12]. It was also found to inhibit retinoic acid-induced differentiation of F9 cells via recruitment of histone deacetylase 3 complex [13]. On the other hand, JDP2 was found to act as a co-activator for the progesterone receptor [14]. Recently we identified JDP2 as a *bona fide* substrate for JNK [15]. JDP2 is phosphorylated on Thr-148

Abbreviations used: ATF, activating transcription factor; EGF, epidermal growth factor; ERK, extracellular signal-regulated protein kinase; HA, haemagglutinin; GST, glutathione S-transferase; JDP2, c-Jun dimerization protein 2; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEKK, mitogen-activated/extracellular-signal-regulated kinase kinase kinase; wt, wild-type.

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both *in vitro* and *in vivo*. Here we describe the mapping of the JNK-docking domain within JDP2. Although JDP2 contains a putative consensus JNK-docking site located at the leucine zipper motif, this domain does not serve to recruit JNK to JDP2. The novel JNK-docking domain maps C-terminally to the phosphoacceptor site and can direct JNK to an otherwise non-phosphorylated substrate with high efficiency. The novel domain identified, although important for JDP2 phosphorylation by the p38 kinase, is not sufficient to direct phosphorylation by p38 kinase. This indicates that differential targeting determinants are used to direct the various stress activating kinases to JDP2.

MATERIALS AND METHODS

Expression plasmids

Mammalian expression plasmids

Mammalian expression plasmids coding for the following cDNAs fused to a His_6 tag were based on the pcDNA 3.1 expression plasmid (Invitrogen): JDP2 wild-type (wt), JDP2Ala ($T^{148}A$, where Thr-148 has been replaced with an alanine residue), JDP2 ($L^{130}Y$, where Leu-130 has been replaced with a tyrosine residue), JTF3 (JDP2 amino acids 1–143; ATF3 amino acids 153–182). pCan is a pcDNA-based expression vector in which the cDNA is fused to a c-Myc epitope tag. The cDNA coding for ATF-JDP (ATF3 amino acids 1–162 fused to JDP2 amino acids 150–163) was inserted in frame with the Myc epitope tag into the pCan expression plasmid. pGC HA-ATF3 wt, containing a haemagglutinin (HA) epitope, was a kind gift from Dr Tsonwin Hai [16]. ATF3 ($Y^{144}L$) was an HA-ATF3 mutant in which Tyr-144 was substituted with a leucine residue by site-directed mutagenesis of the pGC expression vector.

Retroviral vector pBaBe [17] was used to produce stable cell lines with the following plasmids: pBaBe-JDP2 wt, pBaBe-JDP2 ΔC and pBaBe-JDP2 Ala ($T^{148}A$).

Srα-based mammalian expression plasmids encoding for the HA tag, HA-JNK2, HA-ERK2 and activated mitogen-activated/extracellular-signal-regulated kinase kinase kinase (MEKK) were described previously [18,19].

Bacterial expression plasmids

The following plasmids were constructed for expression of bacterial histidine-tagged proteins based on pET15b (Novagen): JDP2 wt, JDP2 Ala ($T^{148}A$) and JDP2 ($L^{130}Y$) (the histidine tag was taken from pcDNA, which has a longer histidine tag than pET15b). The following plasmids were constructed to express glutathione S-transferase (GST) fusion proteins in a pGEXkg-based vector: JDP2 wt, JDP2 ΔC (JDP2 amino acids 1–153), JDP2-Dock (132–163), ATF3 wt and ATF-JDP (ATF3 amino acids 1–162 fused to JDP2 amino acids 150–163). All deletions and chimaeras were constructed using PCR with appropriate primers by generation of specific restriction-enzyme recognition sequences. All constructs were verified by sequencing and found to be free of PCR mutations.

Cell culture and transfection

Mouse fibroblast cells (NIH3T3), human epithelial cells (HeLa) and HEK-293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 4.5 g/ml D-glucose and 10% fetal calf serum. Cells were plated on to 60 mm dishes (3×10^5 cells/dish) and were transfected the following day with 3 μg of DNA using X-tremeGene reagent (Roche) according to the manufacturer's instructions. Following transfection (40 h), cells were harvested and nuclear extract was prepared [20]. Cells that were serum starved for 24 h were stimulated with PMA (50 ng/ml) or anisomycin (50 ng/ml), or irradiated with UV (40 J/m^2) and harvested 30 min after stimulation.

Stable transfection

NIH3T3 cells were infected with replication-defective retroviruses to generate cell lines overexpressing different JDP2 mutants. Retroviruses were generated by transfection into a viral packaging cell line 293gp as described previously [17]. Infected cells were selected with puromycin (3 μg/ml) and pooled following 2 weeks of selection.

Western blot analysis

Western blot analysis was performed as described previously [15]. The following polyclonal antibodies were used: anti-ATF3 (Santa Cruz Biotechnology), anti-c-Jun (Santa Cruz Biotechnology) and anti-JDP2 [9]. The following monoclonal antibodies were used: anti-HA (12CA5) and anti-Myc (9E11; both from Babco).

Kinase assay

NIH3T3 cells were transfected with the Srα plasmid coding for HA-JNK2 or HA-ERK2 as described in [15]. *In vitro* kinase assays were performed as described in [18]. Purified recombinant active p38α (F327S) was obtained from Dr D. Engelberg [21]. Phosphorylation of substrate proteins was examined, following SDS/PAGE, by autoradiography, and quantification was by PhosphorImaging.

Protein expression and purification

GST and histidine fusion proteins were expressed in the *Escherichia coli* BL21 strain and purified using glutathione–Sepharose beads (Sigma) or Ni^{2+}–Sepharose beads (Qiagen) respectively, according to the manufacturers' instructions. The purified proteins were dialysed against PBS containing 10% glycerol.

RESULTS

ATF3 is not a JNK substrate

The human ATF3 is a basic leucine zipper family member exhibiting 61% amino acid homology with human JDP2. The basic leucine zipper motif is the most conserved region (90% homology), whereas lower conservation exists at the N- and C-terminal regions. Interestingly, ATF3 protein derived from various species contains a putative phosphoacceptor site at an identical position when compared with JDP2 Thr-148 (Figure 1A). Since ATF3 is an immediate early gene that is highly induced in response to various stress stimuli [22,23], we tested whether or not ATF3 is phosphorylated by JNK. We transfected HEK-293 cells with plasmids encoding HA-tagged ATF3 and followed the migration of ATF3 protein using SDS/PAGE and Western blotting probed with anti-HA antibody. Transfected cells were either left untreated or treated with PMA (an ERK activator) or anisomycin (an activator of stress-activating kinases, p38 and JNK) prior to cell extraction. Following these treatments no change in ATF3 migration was observed (Figure 1B), suggesting that ATF3 was not phosphorylated in response to activation of all three MAPKs.

ATF3 expression levels are undetectable in growing cells and its levels are highly induced upon serum stimulation [22,23]. Therefore, in order to follow the ability of the endogenous ATF3

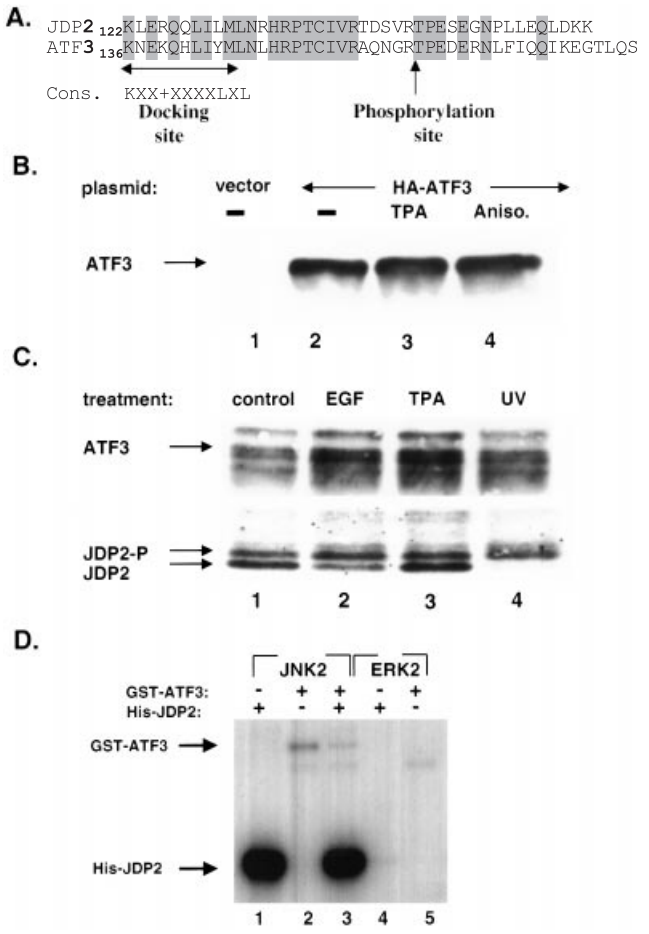

Figure 1 ATF3 is not phosphorylated by JNK

(**A**) Amino acid sequence comparison between JDP2 (amino acids 122–151) and ATF3 (amino acids 136–165). The shaded areas correspond to identical residues. The consensus (Cons.) JNK-docking domain and the proline-directed phosphoacceptor site are indicated. The + sign represents a positively charged residue. (**B**) HEK-293 cells were transfected with PCG5-HA-ATF3 and either left untreated or stimulated with PMA (TPA) or anisomycin (Aniso.) prior to cell harvesting. Nuclear extract was resolved by SDS/PAGE (12% gel) followed by Western blotting with anti-HA antibody. The migration of HA-ATF3 is indicated. (**C**) HeLa cells were stimulated with 20% serum 3 h prior to exposure to the following treatments 30 min prior to cell harvesting: untreated (control), EGF, PMA (TPA) and UV irradiation (UV). Western blotting was performed as described in (**B**) with either anti-ATF3 (top panel) or anti-JDP2 (bottom panel) antibodies. The migration of ATF3, JDP2 and phosphorylated JDP2 (JDP2-P) is indicated. (**D**) Bacterially purified ATF3 is not phosphorylated by JNK2; 10 μg of purified GST-ATF3 or His-JDP2 were incubated with immunopurified activated kinases (either HA-JNK2 or HA-ERK2) in the presence of [γ-^{32}P]ATP for 30 min and separated by SDS/PAGE (12% gel), dried and exposed to autoradiography. The Western blots presented in (**B**)–(**D**) are representative of at least three independent experiments.

to serve as a JNK substrate, serum-starved HeLa cells were exposed to 20% serum for 3 h and either left untreated or stimulated by epidermal growth factor (EGF), PMA or UV irradiation. Cell extract was separated by SDS/PAGE followed by Western blotting and probed with anti-ATF3 antibody (Figure 1C, top panel). As previously reported, ATF3 migrates as a 21 and 23 kDa doublet band due to an alternative ATG usage and not due to phosphorylation [24]. Following EGF and PMA treatments, no change in the mobility of ATF protein was detected. In contrast, reprobing the filters with anti-JDP2 antibodies revealed the typical supershift in JDP2 migration following activation of the stress-activated MAPKs by UV irradiation (Figure 1C, bottom panel).

To ascertain that ATF3 cannot serve as a JNK substrate, we purified GST-ATF3 protein from bacteria and incubated the protein *in vitro* with the immuno-purified activated kinases HA-JNK2 or HA-ERK2 in the presence of [γ-^{32}P]ATP (Figure 1D). Whereas His-tagged JDP2 was efficiently phosphorylated by JNK2 but not ERK2, ATF3 showed no phosphorylation by either of them. In addition, preincubation of ATF3 and JDP2 proteins did not result in ATF3 phosphorylation, nor did it affect JDP2 phosphorylation. Thus these results strongly suggest that, although the phosphoacceptor site exists in ATF3, it cannot undergo phosphorylation by JNK.

The putative JNK-docking domain within the JDP2 leucine zipper domain is not functional

One possible explanation for the inability of ATF3 to serve as a JNK substrate may be due to the lack of a JNK-docking site. In JDP2 a putative perfectly matched JNK-docking consensus sequence is situated 15 amino acids upstream of Thr-148 located within the leucine zipper motif. Indeed, in ATF3 the consensus sequence deviates only by one amino acid, at the core LXL motif (Figure 1A).

To test the possibility that the putative docking domain can function within the leucine zipper motif, we decided to substitute Leu-130 with a tyrosine residue (Figure 2A, JDP2 L^{130}Y). Bacterially purified His-tagged JDP2 L^{130}Y was used in an *in vitro* kinase with immunopurified JNK2. JDP2 L^{130}Y was phosphorylated by JNK2 as efficiently as the wild-type protein (Figure 2B). The difference in migration observed between the wild-type JDP2 and L^{130}Y mutant proteins is due to the different length of the His tag used to generate JDP2 L^{130}Y (see the Materials and methods section). Conversely, we substituted Tyr-144 in ATF3 for a leucine residue (Figure 2A, ATF3 Y^{144}L). This resulted in a perfect match with the JNK-docking consensus within the leucine zipper domain and upstream of the putative phosphoacceptor site. HA-ATF3 Y^{144}L was expressed in NIH3T3 cells that were either left untreated or UV-irradiated prior to cell extraction followed by SDS/PAGE and Western blotting with anti-HA antibody. The ATF3 Y^{144}L mutant protein migrated as a single distinct band, whereas endogenous c-Jun migrated more slowly in cell extracts derived from UV-irradiated cells (Figure 2C). This result indicates that, although a consensus JNK-docking domain was located upstream of the ATF3 phosphoacceptor site, it was unable to confer its phosphorylation. This may be due to the fact that the docking domain is situated within the ATF3 leucine zipper domain and thus was unable to efficiently recruit JNK to the ATF3 putative phosphoacceptor site (Figure 2C). This result suggests that JDP2 may recruit JNK via an alternative docking site.

JDP2 C-terminal domain is responsible for JNK phosphorylation

To map the JNK-docking domain, we used the paradigm of the two homologous proteins and generated the following protein chimaera in which the C-terminal domain of ATF3 (amino acids 158–182) was fused to JDP2, downstream of the putative JNK-docking site (amino acid 143), designated JTF3 (Figure 3A). The different JDP2 proteins (either wild-type or mutant) were expressed in NIH3T3 cells and either left untreated or exposed to UV irradiation followed by cell extraction, SDS/PAGE, Western blotting and probing with anti-JDP2 antibody (Figure 3B). Whereas JDP2 wild-type protein was efficiently phosphorylated following UV irradiation, as observed by the appearance of a lower-mobility species, the JTF3 chimaeric protein migrated as

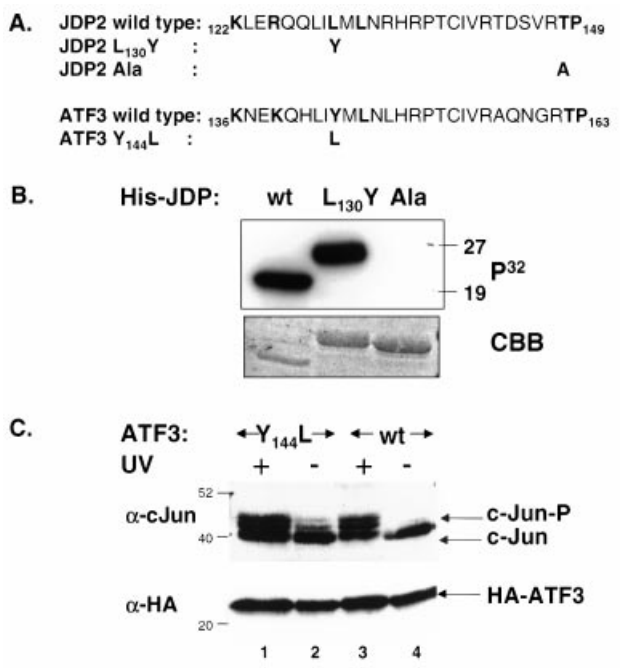

Figure 2 Putative JNK-docking site does not function within the leucine zipper motif

(**A**) Amino acid sequence of the various mutants used. Letters in bold represent the amino acids that constitute the putative consensus docking and phosphoacceptor site. (**B**) Substitution of Leu-130 with tyrosine did not affect JDP2 phosphorylation by JNK. Bacterially purified His-JDP2 wild-type and mutants were incubated with immunopurified activated HA-JNK2 in the presence of [γ-^{32}P]ATP for 30 min and separated by SDS/PAGE (12% gel), dried and exposed to autoradiography. The migration of JDP2 L^{130}Y and JDP2 T^{148}A (Ala) was slower compared with wild-type JDP2 (wt) due to a longer His tag used to generate the chimaeric gene. (**C**) Converting the ATF3 putative JNK-docking domain to a JNK consensus motif did not result in ATF3 phosphorylation. NIH3T3 cells were transfected with either HA-ATF3 wild-type or mutant ATF3 Y^{144}L expression plasmid and either left untreated (−) or UV-irradiated (+). Nuclear extract was prepared and treated as described in Figure 1(B). Western blot was probed with either anti-HA (bottom panel) or anti-c-Jun (top panel). The slower-migrating species observed with anti-c-Jun following UV irradiation represents the phosphorylated form of c-Jun. The Western blots presented in (**B**) and (**C**) are representative results from at least four independent experiments. CBB, Coomassie Brilliant Blue.

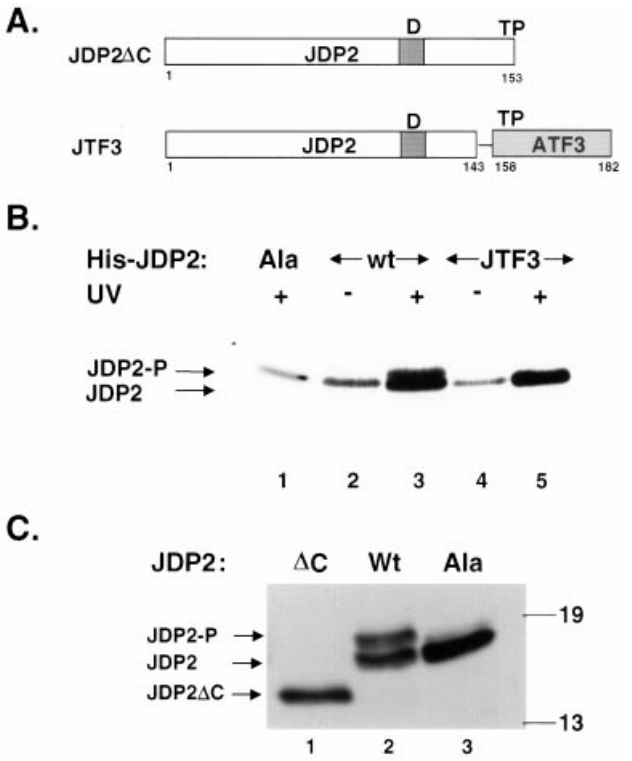

Figure 3 JDP2-ATF3 chimaera is not phosphorylated by JNK

(**A**) Schematic diagram representing the deletion mutant and chimaeric proteins used: JDP2 lacking amino acids 154–163 (JDP2 ΔC) and JDP2 in which the C-terminal domain was replaced with the ATF3 C-terminal domain (155–182) designated JTF3. The D domain is indicated by a grey box. Hatched and blank boxes represent ATF3 and JDP2 protein fragments respectively. TP, Thr-Pro. (**B**) Various JDP2 wild-type and mutant expression plasmids were transiently transfected into the NIH3T3 cell line and nuclear extracts were resolved by SDS/PAGE probed with anti-JDP2 antibodies. Cell extract was derived from untreated (−) or UV-irradiated (+) cells. Ala represents JDP2 T^{148}A. (**C**) The JDP2 C-terminal domain is necessary for JDP2 phosphorylation. NIH3T3 stable cell lines expressing wild-type JDP2 (Wt), JDP2 T^{148}A (Ala) or JDP2 ΔC (ΔC) stable cell lines were UV-irradiated prior to cell extraction and separation by SDS/PAGE and Western blotting with anti-JDP2 antibodies. The migration of JDP2, phosphorylated JDP2 and JDP2 ΔC is indicated. The Western blots presented in (**B**) and (**C**) are representative of at least three independent experiments.

a single band (Figure 3B lane 5), suggesting that JTF3 was not phosphorylated in response to UV irradiation.

This result suggests that the C-terminal domain of JDP2 is responsible for its phosphorylation by JNK. However, the possibility that the ATF3 C-terminal domain exhibits some negative constraint which prevents its phosphorylation could not be excluded. Therefore we truncated the last 10 amino acids of JDP2 (Figure 3A, JDP2 ΔC) and examined the phosphorylation state of different JDP2 wild-type and mutant proteins in the corresponding NIH3T3 stable cell lines following UV irradiation (Figure 3C). Whereas wild-type JDP2 displayed a slower-migrating band, both JDP2 T^{148}A and JDP2 ΔC proteins migrated as single bands (Figure 3C), suggesting that the C-terminal domain is necessary for JDP2 phosphorylation on Thr-148.

The JDP2 C-terminal domain is sufficient for JNK recruitment

The above results suggest that the JDP2 C-terminal domain exerts a positive effect for its phosphorylation and argue against a negative effect resulting from fusion of the ATF3 C-terminal fragment. To test this more directly, we fused a 14-amino acid fragment derived from the JDP2 sequence (amino acids 150–163), located downstream of the Thr-148 phosphoacceptor site, to the c-Myc epitope tag of ATF3, downstream of the putative threonine phosphoacceptor site (Figure 4A, ATF-JDP). The ATF–JDP chimaera generated was expressed in NIH3T3 cells that were: left untreated, UV irradiated or co-transfected with an expression plasmid encoding for truncated MEKK (MEKKΔ). Protein migration was examined using Western blot analysis with anti-Myc antibody. Indeed the ATF-JDP chimaera exhibited two slower-migrating species in cell extracts derived from both UV-irradiated cells and MEKKΔ-cotransfected cells (Figure 4B), indicative of efficient hyper-phosphorylation in response to JNK activation. The ATF3 protein sequence consists of additional two potential JNK phosphoacceptor sites, Ser-24 and Thr-38, and therefore may be phosphorylated as well in the ATF-JDP chimaera. Cell extract treated with potato acid phosphatase generated fast-migrating species typical to the non-phosphorylated form (results not shown).

To demonstrate that the C-terminal domain plays a major role in the recruitment of JNK to JDP2, we fused GST to various cDNA fragments encoding: the full-length JDP2 protein (GST-JDP2), JDP2 with a C-terminal truncation (GST-JDP2 ΔC), the

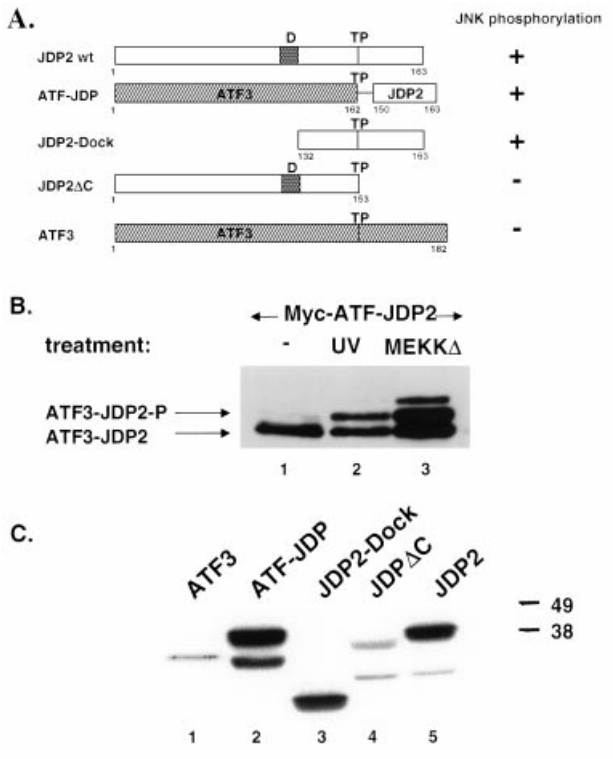

Figure 4 The JDP2 C-terminal domain is able to recruit JNK

(**A**) Schematic diagram representing the deletion mutant and chimaeric proteins used. The D domain is indicated by a grey box. Hatched and blank boxes represent ATF3 and JDP2 protein fragments respectively. TP, Thr-Pro. (**B**) NIH3T3 cells were transfected with an expression plasmid encoding the Myc-epitope-tagged ATF-JDP chimaera and either left untreated (—) or UV-irradiated (UV) 30 min prior to cell harvesting. The cell extract in lane 3 was co-transfected with Srα expression plasmid encoding activated MEKK (MEKKΔ). Nuclear extract was separated by SDS/PAGE probed with an anti-Myc monoclonal antibody (9E11). The migration of the ATF–JDP chimaera as well as the slower-migrating phosphorylated form is indicated. (**C**) The indicated bacterially purified GST fusion proteins were incubated with immunopurified HA-JNK2 and were incubated in the presence of [γ-^{32}P]ATP for 30 min. Proteins were separated by SDS/PAGE (10% gel), dried and exposed to autoradiography. The Western blots presented in (**B**) and (**C**) are representative of at least three independent experiments.

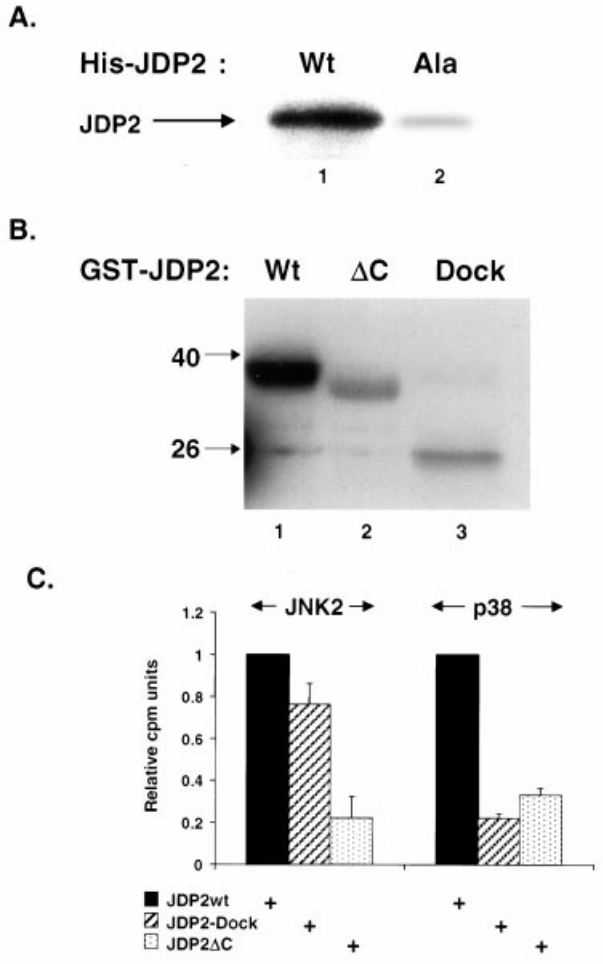

Figure 5 JDP2 C-terminal domain is necessary but not sufficient for p38 phosphorylation

(**A**) p38 phosphorylated JDP2 at Thr-148. Bacterially purified His-JDP2 (Wt) or His-JDP2 T^{148}A (Ala) were incubated with bacterially purified activated p38 F^{327}S [21] in the presence of [γ-^{32}P]ATP for 30 min. Proteins were resolved by SDS/PAGE (12% gel), dried and exposed to autoradiography. (**B**) The JDP2 C-terminal domain is necessary but not sufficient for phosphorylation by p38 kinase. Bacterially purified GST fusion proteins with full-length JDP2 (Wt) C-terminally truncated JDP2 (ΔC) and JDP2 C-terminal fragment (Dock) were used in an *in vitro* kinase assay as described in (**A**). A representative experiment is presented. (**C**) *In vitro* kinase assay using GST-JDP2 (JDP2wt), JDP2 ΔC and JDP2-Dock as substrates with either activated p38 or HA-JNK2 kinases. Protein mixtures were resolved by SDS/PAGE, fixed, dried and analysed by PhosphorImaging. The results represent means $\pm$ S.E.M. from three independent experiments.

JDP2 C-terminal domain consisting of amino acids 132–163 (JDP2-Dock) or a ATF3 chimaera with the JDP2 C-terminal domain (ATF-JDP; Figure 4A). The chimaeric proteins were purified from bacteria and incubated *in vitro* with bacterially purified activated JNK in the presence of [γ-^{32}P]ATP, followed by SDS/PAGE, gel drying and exposure to autoradiography (Figure 4C). Consistent with the results obtained in the NIH3T3 cell line, the ATF-JDP chimaera was efficiently phosphorylated by JNK *in vitro* (Figure 4C). In addition, the GST-JDP2-Dock protein was phosphorylated at a similar level to that of the wild-type JDP2. Both ATF3 and JDP2 ΔC were poorly phosphorylated by JNK *in vitro* (Figures 4C and 5C).

Differential recruitment of the stress-activated kinases by the JDP2 C-terminal domain

JDP2 was shown to be phosphorylated by the stress-activated kinase, JNK. Since both JNK and p38 are activated upon similar stimuli, we tested whether or not p38 is able to phosphorylate JDP2 using an *in vitro* kinase assay (Figure 5A). Although purified wild-type JDP2 exhibited efficient phosphorylation by the p38 kinase, the mutant JDP2 T^{148}A did not incorporate labelled phosphate in the presence of activated p38 kinase. This indicates that both p38 and JNK kinases are able to integrate stress signals to JDP2 Thr-148.

To test whether p38 is recruited to JDP2 via similar sequences located at the C-terminus, we performed *in vitro* kinase assays with different GST-JDP2 fusion protein fragments and bacterially purified activated p38 mutant kinase [21]. This analysis revealed that deletion of the C-terminal region of JDP2 abrogated its phosphorylation by p38 kinase (Figures 5B and 5C), suggesting that the JDP2 C-terminal region is important for the recruitment of both p38 and JNK kinases. However, in contrast to the ability of the JDP2-docking domain (amino acids 132–163) to undergo phosphorylation by JNK (Figures 4C and 5C), the bacterially purified GST-JDP2-Dock did not undergo phosphorylation by

activated p38 kinase (Figures 5B and 5C). This result indicates that p38 and JNK are targeted to JDP2 via distinct docking domains.

DISCUSSION

The MAPKs amplify and integrate signals from a variety of stimuli to produce specific cell responses. To ensure correct cell function to distinct signals, multiple mechanisms have evolved. One mechanism by which MAPKs maintain specificity is by physically associating with their substrates, regulators and upstream kinases via docking sites [2,3]. The best-characterized MAPK-docking domain, common to ERK, JNK and p38 kinases, is the D domain composed of basic residues and a hydrophobic motif separated by 2–6 residues. Both elements are vital for the association and phosphorylation of the MAPK-binding proteins [25]. Other motifs were identified for ERK, such as the FXFP motif identified originally in Lin-1 [26] and the LAQRR motif found in ribosomal S6 kinase and MAPK-interacting kinase [27,28]. A similar motif was found for MAPK-activated protein kinase specific for the p38 kinase [27], yet no similar motifs were identified for JNK. Here we describe the mapping of a JNK-docking domain within the JDP2 substrate.

Based on sequence comparisons with other JNK substrates, a consensus D domain is found within the JDP2 leucine zipper motif. Among all JNK substrates for which the docking domain was mapped, none of the motifs were found to be involved simultaneously in association with other proteins [3]. Since the leucine zipper motif is involved in homo- and heterodimerization with other family members, it seemed unlikely that this docking motif could function as such. Indeed, we found that this domain is unable to recruit JNK to JDP2. Mutations that disrupt the LXL motif had no effect on JDP2 phosphorylation (Figure 2B). Conversely, converting the defective docking site of ATF3 to fulfil the JNK consensus motif did not result in ATF3 phosphorylation by JNK (Figure 2C). Moreover, a JDP2 fragment devoid of the leucine zipper motif and the consensus D domain was efficiently phosphorylated by JNK (Figures 4C and 5C).

Interestingly, the novel docking domain responsible for recruitment of JNK was mapped to the C-terminal domain of JDP2 (Figures 3 and 4), located C-terminally to the Thr-148 phosphoacceptor site. This is the first demonstration of a JNK-docking domain to be localized C-terminally to the phosphoacceptor site. The precise amino acids involved in docking JNK to JDP2 have yet to be determined. Once identified, this would enable the location of the docking motifs of known substrates as well as identification of novel putative JNK substrates.

One substrate, multiple kinases

Although various substrates are phosphorylated specifically by a unique member of the MAPK family, some substrates are phosphorylated by multiple kinases. These include ELK1/TCF1, for example, which recruits both ERK and JNK kinases [29]. Likewise, we described that stress signals mediated by the two stress-activated kinases p38 and JNK are integrated into phosphorylation of JDP2 at Thr-148. Mapping the JNK-docking domain within JDP2 revealed that the C-terminal domain is both necessary and sufficient for Thr-148 phosphorylation. However, this domain is only necessary for phosphorylation by the p38 kinase but is not sufficient to confer phosphorylation of Thr-148. This suggests that additional sequence determinants, yet to be identified, are required for phosphorylation by the p38 kinase.

The kinase common docking motif

Recently a motif containing critical acidic residues was identified in ERK, JNK and p38 MAPKs, which acts as a docking site for binding to different MAPK substrates, designated the common docking domain [30]. The common docking motif is located in the C-terminal region outside the catalytic domain, while a second, the ED motif (Glu-Asp), contributes to substrate specificity [31]. The binding of this domain is mediated via association with basic residues found in the substrate-docking domains [25].

The JNK common docking domain consists of only three negatively charged residues, as opposed to those of ERK and p38, which are composed of 5–6 negatively charged residues. The fact that the JDP2 C-terminal docking domain contains two positively charged residues may be more favourable for JNK binding, whereas the p38 kinase requires additional positively charged residues that are located upstream of the JDP2 phosphoacceptor site to neutralize its bulk negatively charged residues. We observed that JDP2 could compete for c-Jun and ATF2 phosphorylation *in vitro* (S. Katz and A. Aronheim, unpublished work), suggesting that a common docking domain in JNK is used for binding the distinct docking domains found in ATF2, c-Jun and JDP2.

ATF3 phosphorylation

Based on the sequence conservation of the putative phosphoacceptor site in the ATF3 proteins derived from different species, one would expect that this site might be regulated similarly to JDP2. However, in contrast to JDP2, whose protein levels are constantly and ubiquitously expressed, ATF3 is found in very low abundance in resting cells but its levels are highly induced upon stress signals. Thus ATF3 activity is mainly regulated at the transcriptional level, and when maximum protein levels are reached the stress-activated kinases are no longer active and therefore phosphorylation by these kinases is no longer required for modulation of its activity.

Although ATF3 is phosphorylated in adenovirus-E1-transformed cells [24] the kinase responsible for its phosphorylation is not known. We could not detect ATF3 phosphorylation as a result of JNK and/or p38 activation *in vivo* or *in vitro*; however, we cannot exclude the possibility that, under different cell stimuli or physiological circumstances, ATF3 is phosphorylated by the stress kinases.

Multiple substrate-docking domains

It is becoming apparent that, apart from the existence of common basic residues within docking domains found in different JNK substrates, they share little sequence homology. The precise physiological roles of the variable determinants for JNK substrates have yet to be identified. One possibility could be the fact that JNK plays a role in multiple and diverse cell functions. Towards this end, phosphorylation of a different subset of substrates is necessary. Although different affinities towards various substrates have been found *in vitro* [26], so far such a phosphorylation hierarchy has not been described *in vivo*. Nevertheless, further studies towards identification of additional JNK substrates and mapping of their docking domains would enable examination of this hypothesis.

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