The JunD transcription factor is one member of the Jun family of proteins that also includes c-Jun and JunB. Although c-Jun can function to promote cell proliferation and can cooperate with other oncoproteins to transform cells, JunD slows proliferation of fibroblasts and antagonizes transformation by activated ras. Two isoforms of JunD, a full-length isoform containing 341 amino acids (JunD-FL) and a truncated isoform lacking 48 amino acids at the N terminus (ΔJunD), are generated through utilization of two translation start sites within a single mRNA. Here we show that both isoforms of JunD are phosphorylated by Jun N-terminal kinases (JNKs) at three identical residues and that both contain a docking domain that specifically binds JNKs. The JunD-FL isoform binds to and is phosphorylated by JNK more efficiently than ΔJunD in vitro; correspondingly, JunD-FL is a more potent transcriptional activator than ΔJunD. Although increased JNK signaling can activate both JunD isoforms, mutating either the JNK docking domain or the target JNK phosphorylation sites blocks this activation. These results identify two distinct isoforms of JunD with differential responses to JNK signaling pathways.

The AP-1 transcription factor consists of a large set of dimer combinations formed between the Jun, Fos, and ATF families of proteins (1–3). The Jun family, consisting of c-Jun, JunB, and JunD, can form both heterodimers and homodimers among themselves, whereas Fos family members (c-Fos, FosB, Fra1, and Fra2) can only heterodimerize with Jun proteins (4–8). AP-1 activity converts extracellular signals into changes in gene expression patterns through the binding of AP-1 dimers to specific target sequences located within the promoters and enhancers of target genes. These targets include genes important for regulating many biological processes including proliferation, differentiation, apoptosis, and transformation (2, 9–11). AP-1 activity functions in a hierarchy; dimerization of AP-1 proteins is required for DNA binding that in turn leads to transcriptional activity (2). Although the three Jun proteins share a high level of sequence and functional homology, they exhibit distinct expression patterns during development and differ in their transcriptional and biological activities (12, 13). For example, although c-Jun can transform cultured cells and can cooperate efficiently with ras in transformation, JunD inhibits its fibroblast proliferation and antagonizes transformation by ras (14–16). Each Jun also has distinct roles in mouse development with c-Jun and JunB required for viability (17–19).

Regulation of the Jun proteins occurs at the level of transcription and through post-transcriptional modifications, primarily phosphorylation. A subgroup of the MAP kinase family that helps to regulate Jun and AP-1 activity is that of the Jun N-terminal kinases (JNKs). JNKs are known to phosphorylate residues located in the N-terminal region of c-Jun and activate its transcription (20–23). Phosphorylation of two sites (serine 63 and serine 73 in the mouse) is essential for stress-induced apoptosis and oncogenic transformation by ras and fos (24–26). Phosphorylation of the N terminus of c-Jun increases its transcriptional activity by altering its interaction with other nuclear proteins including co-activators like the CREB-binding protein (27, 28). Efficient phosphorylation of these sites requires the structural integrity of a JNK-binding domain located adjacent to the target residues (29, 30). The specificity of JNK and other MAP kinase signaling is conveyed, in part, by the binding of kinases to “docking” sequences present in the proteins they interact with. These interacting proteins include upstream MAP kinase kinases, phosphatases, substrate proteins (e.g., transcription factors), and scaffold proteins (31–34). The docking sequences are highly conserved and modular, allowing the identification of consensus sequences for particular MAP kinase families that are useful for functional analyses and for finding novel MAP kinase-interacting proteins.

JNK phosphorylation target sites are present in the JunD protein, and phosphorylation of JunD by JNK has been detected in several studies (16, 30, 35, 36). However, the interactions of JNK and JunD have not been studied closely. Two distinct isoforms of JunD are ubiquitously expressed, JunD full-length (JunD-FL) and ΔJunD (37–39). These two JunD isoforms are generated by the use of two highly conserved translation start codons within a single JunD mRNA, and the two proteins differ only in a 48-amino acid N-terminal extension present in the mouse JunD full-length protein (43 amino acids in the human protein). Both JunD isoforms retain prospective JNK target sequences. Here we report that JunD-FL and ΔJunD are phosphorylated by JNK at the same three residues but with different efficiencies. Further, we have characterized a functional JNK docking domain present in both JunD isoforms and identified amino acid residues that are essential for JNK docking activity. These similarities and differences in the interactions of JunD-FL and ΔJunD with JNKs are likely to be reflected in distinct transcriptional and biological properties between these two proteins.
EXPERIMENTAL PROCEDURES

**Cell Culture**—Chinese hamster ovary (CHO) cells were grown in Ham’s F-12 medium (CellGro) supplemented with 5% fetal bovine serum (HyClone Laboratories, Inc.) and penicillin/streptomycin (BioWhittaker) under standard conditions. Recombinant IL-1β was purchased from Upstate Biotechnology, Inc.

**Expression Vectors**—Expression vectors for GST fusion proteins were prepared using the pGEX4T-3 vector (Pharmacia Corp.). Insert DNA encoding the N-terminal fragments of c-Jun (amino acids 1–123), JunD (amino acids 1–149 and 1–115), and ΔJunD (amino acids 49–149 and 49–115) with BamHI and EcoRI restriction sites at their 5′ and 3′ ends, respectively, were generated using PCR from cDNA templates. The inserts were gel-purified, digested, and ligated into pGEX4T-3 cut with the same enzymes. The expression vector for GST-c-Jun (1–79) was provided by Dr. G. Kraft. Expression vectors for the GAL4 fusion proteins were constructed using pGAL0 that is a modified version of the pSG424 plasmid (40). Insert DNA containing the same Jun N-terminal fragments indicated above were generated by PCR. Restriction enzyme sites for BamHI and XbaI at the 5′ and 3′ ends, respectively, were included in the primer design. The PCR products were gel-purified, digested, and ligated into pGAL0 cut with the same enzymes. All of the constructs were verified by sequencing using an ABI automated sequencer. The pSR-α-HA-JNK1 and pSR-α-HA-JNK2 expression plasmids were obtained from Dr. A. Lin (41). Additional JNK expression plasmids (pcDNA3-JNK2a1, JNK2a2, JNK2b2, JNK1a1, and JNK3a1) were obtained from Dr. R. Davis (41). Plasmid DNA stocks used for transfections were prepared using a standard cesium chloride density gradient procedure or Maxiprep kits (Qiagen).

**Bead Assays**—GST fusion proteins were expressed in *Escherichia coli* DH5α-F′ (Invitrogen) using a standard protocol. The cells were lysed in GST lysis buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.1% β-mercaptoethanol, 5 µg/ml each of aprotonin, leupeptin, and pepstatin, 1 mM phenylmethylsulfonyl fluoride, and 1 mg/ml lysozyme; Sigma). The cells were lysed by pipetting repeatedly. Triton X-100 was added to a final concentration of 1% after lysis. The lysate was clarified by centrifugation at 14,000 × g for 5 min. The clarified GST-fusion protein was then incubated in SDS sample buffer, boiled for 5 min. Whole cell extracts were fractionated by 12% SDS-PAGE and transferred to nitrocellulose (Schleicher & Schuell), and the complexes were detected and quantified as described in Materials and Methods.

**Immunoprecipitation**—CHO cells were plated at a density of 40,000 cells/cm² 24 h before transfection. 0.3 µg of the reporter plasmid G-E1b-Luc along with 2 µg of pGAL4-Jun encoding each of the GAL4-Jun fusion proteins, and when indicated, 25 ng of pSR-α-HA-JNK1 and 13 ng of pSR-α-HA-JNK2 were transfected into the cells using Cytofectene reagent (Bio-Rad) in serum-free medium according to the manufacturer’s instructions. At 24 h post-transfection, cells were harvested by incubation in passive lysis buffer (Promega) for 30 min at room temperature with agitation. The luciferase activity in the extracts was measured in a Turner Designs luminometer (model 20/20) using a luciferase assay system (Promega) as per the manufacturer’s instructions.

**Western Blot Analysis**—CHO cells were transfected with 0.25 µg of the expression plasmids in 24-well plates. After 24 h, they were rinsed twice with phosphate-buffered saline and harvested in SDS sample buffer, boiled for 5 min. The amount of35S-labeled JNK associated with the GST-Jun proteins was quantified by PhosphorImager analysis. For the kinase assays, the amount of equimolar amounts of substrate proteins. For kinase assays using free peptides, 0.15 µg of JunD-FL (1–115) and 0.09 µg of ΔJunD (49–115) were used. Substrate proteins were mixed with 0.2 µl of TNT lysate supplemented with cDNA for JNK associated with the GST-Jun proteins was quantified by PhosphorImager analysis. For the kinase assays, the reactions were performed in a total volume of 20 µl using ~0.5 µg of each substrate GST fusion protein (corrected for molecular mass to yield equimolar amounts of substrate proteins). For kinase assays using free peptides, 0.15 µg of JunD-FL (1–115) and 0.09 µg of ΔJunD (49–115) were used. Substrate proteins were mixed with 0.2 µl of TNT lysate supplemented with cDNA for JNK associated with the GST-Jun proteins was quantified by PhosphorImager analysis. 0.2 µl of the reporter plasmid G-E1b-Luc along with 2 µg of pGAL4-Jun encoding each of the GAL4-Jun fusion proteins, and when indicated, 25 ng of pSR-α-HA-JNK1 and 13 ng of pSR-α-HA-JNK2 were transfected into the cells using Cytofectene reagent (Bio-Rad) in serum-free medium according to the manufacturer’s instructions. At 24 h post-transfection, cells were harvested by incubation in passive lysis buffer (Promega) for 30 min at room temperature with agitation. The luciferase activity in the extracts was measured in a Turner Designs luminometer (model 20/20) using a luciferase assay system (Promega) as per the manufacturer’s instructions.

**Results**

**Both Isoforms of JunD Are Phosphorylated by JNK in Vitro**—Because the direct interaction of JunD with JNKs has not been studied extensively, we first tested phosphorylation of recombinant JunD proteins by JNKs in vitro. Two forms of JNKs are generated from a single intron-less messenger RNA through use of an alternative translational start site: the full-length isoform (JunD-FL) and the truncated isoform (ΔJunD) that lacks the N-terminal 48 amino acids (Fig. 1A). GST fusion proteins containing N-terminal JunD fragments corresponding to JunD-FL and ΔJunD were purified from bacteria and used as substrates for phosphorylation by JNK2a1. A schematic of the various constructs used in these assays is presented in Fig. 1B. Two fusion fragments were used for each JunD isoform, a short (amino acids 1–115 for JunD-FL and 49–115 for ΔJunD) and a longer fragment (amino acids 1–149 for JunD-FL and 49–149 for ΔJunD). Two similar fusion proteins were prepared for c-Jun and a single fusion protein for JunB. JNK2a1 was prepared by in vitro transcription and translation (TNT; Pro-
mega). As shown in Fig. 2A, both of the longer GST-JunD isoforms (through amino acid 149) were readily labeled, although less rapidly than c-Jun. In addition, JunD-FL was more efficiently phosphorylated than \( \Delta \text{JunD} \). Similar results were obtained when the shorter (through amino acid 115) JunD fusion proteins were tested (Fig. 2B). A slightly larger difference between JunD-FL and \( \Delta \text{JunD} \) was observed with these shorter proteins.

As shown in Fig. 1, the GST moiety in the fusion proteins abuts a prospective JNK docking region in \( \Delta \text{JunD} \). To determine whether this structural arrangement interfered with efficient phosphorylation of the JunD substrate by JNK, free JunD peptides were prepared by cleaving the GST fusion constructs with thrombin. Equal amounts of free JunD peptides were then used in a kinase assay (Fig. 2C). The difference in the rate of phosphorylation between the JunD-FL and \( \Delta \text{JunD} \) peptides was more pronounced than with the intact GST fusion proteins. When the free JunD peptide concentration was lowered in these assays (on a molar basis) compared with the amounts used in the GST-JunD kinase assays (Fig. 2, A and B), phosphorylation appeared to have neared saturation (Fig. 2C). The larger amount of label in the JunD-FL peptide suggested that it contained one or more JNK phosphorylation sites in addition to serine 90 and serine 100. Only one additional prospective JNK/MAP kinase target site (serine or threonine followed by a proline residue ((S/T)P)) was present in the JunD-FL peptide that was not contained in the \( \Delta \text{JunD} \) peptide: threonine 3. Substitution of the threonine 3 residue with alanine had no effect on the rate or extent of phosphorylation of the JunD-FL peptide, indicating that threonine 3 is not a JNK target (data not shown).

**Identification of JNK Target Residues in JunD Isoforms**—To further confirm that there were no additional JNK target sites within the first 48 amino acids of JunD, we compared phosphopeptide maps of the GST fusion proteins that were phosphorylated in vitro by JNK (Fig. 3B). The maps showed similar patterns for both isoforms of JunD, suggesting that they are phosphorylated on identical residues. Moreover, use of the S90A and/or S100A point mutations revealed the positions of the phosphopeptides containing the JNK target sites. Spot c in Fig. 3B is generated from phosphorylation of serine 90, and spots a and b are generated from phosphorylation of serine 90. Lack of phosphorylation of the S90A/S100A double mutant indicated that Ser\(^{90}\) and Ser\(^{100}\) are the only sites that are phosphorylated within these N-terminal fragments (amino acids 1–115) in vitro.

The longer GST-JunD fusion proteins (amino acids 1–149 for JunD-FL and 49–149 for \( \Delta \text{JunD} \)) were also efficient JNK substrates (Fig. 1A). However, when the S90A/S100A double mutation was introduced into the longer GST-JunD (1–149) construct, phosphorylation was not completely abolished. This result suggested the presence of additional JNK target site(s) between residues 116 and 149 (Fig. 3C, lane 4). Sequence comparison between c-Jun and JunD in this region identified a...
likely JunD JNK target residue as threonine 117 (Fig. 3A). Thr₁₁₇ is positioned in a cluster of amino acids (TTPT) that is conserved between JunD and c-Jun and that fits the JNK/MAP kinase consensus. Mutation of threonine 117 to alanine in combination with the S90A and S100A mutations completely abolished phosphorylation of the GST-JunD (1–149) protein (Fig. 3C, lane 3), whereas the single T117A mutation reduced the level of phosphorylation (Fig. 3C, lane 2), and the triple mutation S90A/S100A/S117A abolished labeling (lane 3). WT, wild type.

**Fig. 2.** JunD-FL is phosphorylated more extensively than ΔJunD by JNK2α1 in vitro. A, equal amounts of purified GST-JunD-FL (1–149), GST-JunD (49–149), and GST-c-Jun (1–123) fusion proteins were incubated with in vitro transcribed and translated Jun N-terminal kinase 2α1 (JNK2α1) in the presence of [γ-³²P]ATP. The aliquots were taken at timed intervals, separated by SDS-PAGE, and transferred to nitrocellulose membranes, and the incorporated ³²P signal was detected using a PhosphorImager instrument. The ³²P signals depicted in the upper panel were quantified and plotted against time of incubation. A linear least squares regression fit of the data points was performed, and the slope of each curve is given in parentheses. B, equal amounts of GST fusion proteins containing shorter Jun fragments (through residue 115 for the JunD isoforms and residue 79 for c-Jun) were used in kinase assays as in A. C, free JunD peptides, prepared by thrombin cleavage of GST-JunD fusion proteins, were used for an in vitro kinase assay. Equal amounts of peptides corresponding to the N termini of JunD-FL (amino acids 1–115; 0.15 µg) and to ΔJunD (amino acids 49–115; 0.09 µg) were incubated with equal amounts of JNK2α1 and [γ-³²P]ATP for the indicated times. The reactions were quenched and separated by 17% SDS-PAGE, and the incorporated label was detected as in A. The longer peptide, JunD-FL (1–115), migrates anomalously faster than the shorter ΔJunD peptide. Each kinase assay was repeated at least three times with similar results. A single representative assay is shown for each set of substrates.

**Fig. 3.** Identification of JNK target residues within the JunD sequence. A, alignment of N-terminal regions of mouse JunD and c-Jun sequences showing serine and threonine residues targeted by JNKs. B, phosphopeptide mapping was performed on GST fusion proteins containing residues 1–115 (JunD-FL, left panels) or residues 49–115 (ΔJunD, right panels) that were labeled with [γ-³²P]ATP in vitro using JNK2α1. Mutant proteins were prepared and tested that contained serine to alanine substitutions at the two prospective JNK target residues, serine 90 and serine 100. C, threonine 117 was identified as an additional JNK target residue in JunD. The longer GST JunD fusion protein (amino acids 1–149) containing the S90A and S100A mutations continued to incorporate label in JNK in vitro kinase assays (lane 4). Mutation of threonine 117 to alanine decreased ³²P-labeling (lane 2), and the triple mutation S90A/S100A/S117A abolished labeling (lane 3). WT, wild type.

An Essential Element of the JNK Docking Domain Is Conserved in JunD—A consensus sequence has been derived from
sequence alignment of nine JNK-interacting proteins (44). A cluster of basic amino acids preceding two leucine residues separated by a single amino acid (LXXL motif) has been found to be important functional components of the JNK docking domain (Fig. 5A). Sequence analysis of the three Jun proteins revealed that this motif is conserved in each Jun protein (Fig. 5B). The LXXL motif has been shown to be critical for the interaction of c-Jun with JNK (29). Mutation of the LXXL motif in c-Jun (A40/A42) resulted in an expected loss of JNK-mediated phosphorylation. Mutation of the corresponding residues in JunD-FL and ΔJunD (L57A/L59A) also resulted in the loss of JNK-mediated phosphorylation in vitro kinase assays (Fig. 5C). GST pull-down experiments using these constructs and 35S-labeled in vitro translated JNK2α1 revealed that LXXL mutations in JunD abolished JNK binding (data not shown).

The second important element in the JNK docking domain consensus sequence is the cluster of basic amino acids preceding the LXXL motif. Both c-Jun and JunB contain a perfect consensus sequence and are high affinity substrates for JNKs. JunD deviates from the consensus at the first basic amino acid position (Fig. 5, A and B). We generated a M49K mutation in JunD to match the consensus sequence and a K32M mutation in c-Jun (as a reciprocal control) to determine whether the lack of the basic amino acid at the first position contributed to the observed lower affinity of JNKs for JunD. These mutations did not result in significant changes in the level of JNK binding or JNK-mediated phosphorylation using in vitro assays (Fig. 5, D and E). Furthermore, a GST fusion protein containing a truncated N-terminal fragment of c-Jun (amino acids 32–79), which lacked the N-terminal sequences preceding the characterized JNK docking domain (corresponding approximately to the truncation present in ΔJunD) exhibited significantly lower JNK binding as determined by the GST pull-down assay (data not shown). These results suggest that the N-terminal sequences (amino acids 1–31 for c-Jun and amino acids 1–48 for JunD) are involved in JNK binding.

c-Jun Interferes with JunD-JNK Interaction in Vitro—To test whether the docking domain in JunD is functionally comparable with that of c-Jun, equal amounts of c-Jun and JunD were used in combination as substrates for in vitro kinase
Interactions of JunD Isoforms with Jun N-terminal Kinases

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**A**

| GST-c-Jun | GST-JunD-FL | GST-AlunD |
|-----------|-------------|-----------|
| c-Jun     | WT A40/42   | WT A57/59 | WT A57/59 |
| ΔJunD     |             |           |           |

**B**

| GST-c-Jun | GST-JunD-FL | GST-AlunD |
|-----------|-------------|-----------|
| WT M32    | A40/42      | K49       |

**C**

| GST-c-Jun | GST-JunD-FL | GST-AlunD |
|-----------|-------------|-----------|
| WT M32    | A40/42      | K49       |

**D**

| GST-c-Jun | GST-JunD-FL | GST-AlunD |
|-----------|-------------|-----------|
| WT M32    | A40/42      | K49       |

**E**

| GST-c-Jun | GST-JunD-FL | GST-AlunD |
|-----------|-------------|-----------|
| WT M32    | A40/42      | K49       |

**F**

![Figure 6: c-Jun interferes with interaction of JunD with JNKs in vitro](image)

**Fig. 5. Identification of a functional JNK docking domain in JunD isoforms.** A, two leucine residues separated by a single amino acid (LXL motif) closely following a cluster of basic amino acids are important constituents of the JNK docking domain. B, alignment of the regions of the three Jun members corresponding to the JNK docking domain characterized in c-Jun. Both c-Jun and JunB docking domain sequences closely follow the consensus, and both are known to bind JNKs with high affinity. The LXL motif is conserved in JunD; however, the first amino acid (which is also the translational start site for ΔJunD) in the docking domain region deviates from the consensus. C, the critical leucine residues in the LXL motif in GST-c-Jun (1–79), GST-JunD-FL (1–115), and GST-AlunD (49–115) were mutated to alanines. Equal amounts of purified wild-type (WT) and docking domain mutant proteins were incubated with in vitro transcribed and translated JNK2 and GST-c-Jun proteins were incubated with GST-c-Jun recombinant proteins. E, the same set of GST-JunD proteins used in the JNK pull-down assay in D were used as substrates for in vitro phosphorylation. assays (Fig. 6). The wild-type c-Jun and JunD constructs alone were phosphorylated, and the corresponding LXL mutants were not under these conditions (Fig. 6, lanes 1–4). When the wild-type c-Jun and JunD were incubated together, GST-c-Jun was efficiently phosphorylated, whereas JunD phosphorylation was minimal (Fig. 6, lane 5). Co-incubation of c-Jun-wt and the JunD LXL mutant (A57/A59) resulted in a similar pattern (lane 6). However, JunD-wt was phosphorylated when co-incubated with the LXL mutant of c-Jun (L40A/L42A) (lane 7), and as expected, neither of the LXL mutants was phosphorylated when used in combination (lane 8). These results demonstrate that c-Jun is a higher affinity substrate for the JNKs and that c-Jun can interfere with binding of JNKs to JunD. Furthermore, these data suggest that JNKs bind to comparable sites on both c-Jun and JunD.

**Fig. 6. c-Jun interferes with interaction of JunD with JNKs in vitro.** Equal amounts of purified wild-type (WT) or docking domain mutant proteins were used in an in vitro kinase assay either as a single substrate (lanes 1–4) or in combination (lanes 5–8) as indicated. The substrates were incubated with in vitro transcribed and translated JNK2 and GST-c-Jun proteins were incubated with GST-c-Jun recombinant proteins. The reactions were quenched by heating in sample buffer, the mixtures were separated by SDS-PAGE, and the incorporated 32P signal was detected using a PhosphorImager.

**JNK Docking and Phosphorylation Site JunD Mutants Exhibit Lower JNK Activation in Cultured Cells—To test whether JNKs are important regulators of JunD activity in living cells, we fused N-terminal fragments of JunD (amino acids 1–149 or 49–149) to the DNA-binding domain of the yeast transcription factor GAL4 (Fig. 1C) and tested these fusion proteins for their ability to activate the GAL4 reporter plasmid (G5E1b-Luc) in the presence or absence of ectopic JNK1 and JNK2 expression in transient transfection assays. The wild-type constructs of JunD-FL and ΔJunD yielded significant activation in the presence of overexpressed JNK2 and JNK1 (Fig. 7A). Wild-type JunD-FL exhibited an ~4-fold higher basal activity than ΔJunD, but the ratio of JunD-FL to ΔJunD activity dropped to 2.5-fold after JNK activation. The docking domain mutants (L57A/L59A) of both JunD-FL and ΔJunD showed overall lower activity and reduced activation by JNKs, presumably because of residual binding of JNK to the mutant docking domain. The target site mutants (S90A/S100A) lost activation by JNK completely, indicating that phosphorylation by JNK is essential in the JNK-mediated activation. GAL4-C/EBP(1–97) was used as a negative control, because the C/EBP transcription factor is not a JNK substrate. As expected, GAL4-C/EBP displayed no activation by JNK (data not shown).

To test the involvement of threonine 117 in JunD activity and JNK activation, a similar set of transient transfections were performed (Fig. 7B). Substitution of Thr117 with alanine had no affect on the basal activity of GAL4-JunD (1–149); however, the T117A mutation did attenuate JNK activation. The triple mutant S90A/S100A/117A exhibited no JNK activation at all, similar to the S90A/S100A mutant construct. Expression of the various ectopic proteins was monitored by Western blot analysis (Fig. 7C).

**Phosphorylation of JunD Proteins in Cells—To verify that the GAL4-JunD fusion proteins used in the transfection reporter assays were actually being phosphorylated by JNK in cells, immunoprecipitation-Western blot analysis was performed using antibodies that recognize phosphorylated JunD (Fig. 8). The cells were co-transfected with expression plasmids...
encoding GAL4-JunD, JNK1, and JNK2. To further increase the JNK activity, the cells were treated with IL-1β (30, 41). The GAL4-JunD proteins were immunoprecipitated using an anti-GAL4 antibody, and Western blots of these samples were probed with two different JunD cross-reacting antibodies that were originally prepared against phosphorylated c-Jun peptides. The first antibody (anti-phospho-c-Jun 659; Upstate Biotechnologies, Inc.) was raised against a peptide containing amino acids 70–88 of c-Jun and a phosphorylated JNK target, serine 73. An identical sequence (amino acids 97–109) is present in JunD and includes the JNK target serine-100 (Fig. 3A).

The second antibody was raised against amino acids 57–68 of c-Jun including the JNK target serine 63 (43). A 60% identical sequence is found in JunD (amino acids 84–95) including the JNK target serine 90. Both of these antibodies recognized a pair of slowly migrating bands present in the IL-1β-treated samples. The anti-phospho-c-Jun antibody 659, as expected, exhibited stronger reactivity and also recognized a phospho-specific band in the absence of IL-1β stimulation (Fig. 8A).

**DISCUSSION**

The Jun family of proteins and AP-1 transcriptional activity are implicated in a variety of biological phenomena ranging from cell proliferation and transformation to apoptosis and development (9, 11–13, 45). Although extensively studied, the functional similarities and differences between the three Jun family proteins are not fully characterized. The ubiquitous expression of both JunD-FL and ΔJunD, always at similar stoichiometries, suggests that each isoform possesses unique and conserved functions. On the other hand, because both JunD-FL and ΔJunD are identical but for a 48-amino acid N-terminal extension present in JunD-FL, considerable overlap in function is expected as well. For example, the dimerization and DNA-binding domains of each isoform present in the C-terminal third of the molecules would be expected to confer very similar, if not identical, dimerization and DNA target sequence specificity (46). In this study we have focused on the
N-terminal portion of the JunD molecules, because this is the region structurally different between JunD-FL and ∆JunD. The first 48 amino acids in JunD-FL are rich in glycine and proline residues (11 glycines and 7 prolines); thus, this peptide is expected to be highly unstructured. Interestingly, residues 49–65, which are immediately downstream (that forms the N terminus of ∆JunD), have a high probability of forming an α-helix predicted using the Chou-Fasman algorithm. Thus, a striking structural contrast exists between the N terminus of ∆JunD and the extension present in JunD-FL.

The importance of the N-terminal regions of the two JunD isoforms was recently emphasized by the discovery that the Menin tumor suppressor protein binds to JunD-FL with no detectable affinity for ∆JunD, indicating that the first 48 amino acids in JunD-FL contain the Menin interaction domain (39). The MEN-1 gene encoding Menin was identified as the gene responsible for the disease multiple endocrine neoplasia type 1 (47–49). Binding of Menin to JunD-FL specifically inhibits its transcriptional activation, suggesting a role for JunD-FL in MEN-1 cancers. This differential interaction of Menin is the most significant functional difference identified between JunD-FL and ∆JunD to date and suggests that a balance of activity between the two JunD isoforms may be important in regulating cell proliferation. Residues in JunD-FL have been identified that are important for Menin-JunD-FL binding (50). These residues (Pro41, Gly42, and Pro44) are close to the JNK docking domain that we have identified in this study. This raises the possibility that binding of Menin to JunD may interfere with JNK-JunD interaction and may be one mechanism through which Menin inhibits JunD transcriptional activity.

Phosphorylation of JunD-FL and ∆JunD by JNK—Our results indicate that this flexible N-terminal of JunD-FL enhances the interaction of JunD-FL with JNKs. No predicted JNK docking sequences are present between amino acids 1–48, and no JNK binding in vitro to a GST fusion protein containing these residues was detected (data not shown). Therefore, this region may enhance JNK interaction by affecting the conformation of a downstream JNK docking sequence. Alternatively, residues 1–48 might directly bind to JNK but only in the context of the intact JunD-FL molecule and not as an isolated fragment. Whatever the mechanism, we observed a consistent pattern in the in vitro assays; JunD-FL is phosphorylated more rapidly than ∆JunD. This same pattern was observed when free JunD peptides were used as JNK substrates, indicating that the GST moieties did not interfere with the interaction between JunD and JNK (Fig. 2C). However, both JunD isoforms are less efficient JNK substrates than c-Jun, with JunD-FL incorporating approximately half the 32P label as c-Jun under conditions where the rate of 32P incorporation remained linear for each GST fusion protein tested (Fig. 2, A and B).

The sites of JNK-mediated phosphorylation were directly determined by mutagenesis and phospho-peptide analysis as serine 90, serine 100, and threonine 117 (Fig. 3). Comparison of the c-Jun and JunD sequences between c-Jun residues 58–96 and JunD residues 58–122 revealed 80% identical amino acid residues (Fig. 3A). In particular, serine 90 and serine 100 in JunD corresponding to the known c-Jun phospho-acceptor sites serine 63 and serine 73 are located in a cluster of 95% sequence identity (JunD amino acids 90–109). JunD threonine 117 is positioned in a conserved cluster, TTTPT. Although c-Jun is reported to be phosphorylated on both threonine 91 and threonine 93, JunD lacks the corresponding proline at position 120 that would render threonine 119 a JNK target (51). This is verified by the complete absence of 32P label incorporation in the triple substitution mutant S90A/S100A/S117A (Fig. 3C).

JunD-FL and ∆JunD Bind to JNKs—To directly test the interaction of JunD isoforms and JNKs, pull-down assays were performed using GST-Jun fusion proteins containing fragments of c-Jun, JunD, and JunB. We established that for each JunD isoform tested, the order of binding affinity was c-Jun > JunB > JunD-FL > ∆JunD. The relative JNK affinity of c-Jun compared with JunD-FL ranged from ~2.6-fold for JNK3α1 to 7-fold for JNK1α1 (Fig. 4B). These binding data agree with the in vitro kinase assay results presented in Fig. 2 demonstrating a correlation between JNK affinity and rates of phosphorylation. The relative affinity of c-Jun for JunD compared with JunD was also tested using an in vitro competition kinase assay (Fig. 6). In these experiments when equimolar recombinant c-Jun and JunD-FL proteins were mixed with active JNK, only c-Jun was detectably phosphorylated; however, when the L40A/L42A c-Jun docking site mutant was used, JunD was efficiently phosphorylated. These results suggest that c-Jun and JunD competed for the same binding regions on JNK, consistent with the JunD and c-Jun docking domains being functionally equivalent but with significant differences in affinity toward JNK.

In the GST pull-down assays (Fig. 4) considerable differences in JNK affinity between JunD-FL and ∆JunD were evident between the JNK isoforms. For example, little difference was found between JunD isoforms binding to JNK2β2 versus a 5-fold difference for JNK3α1. Whether these results reflect differences that exist in vivo remains to be demonstrated. However, because JNK3α1 is expressed primarily in the brain, it is possible that tissue-specific regulation of Jun activity may be exerted, at least partially, through differential binding to JNK isoforms.

Both JunD and ∆JunD Are Regulated by JNKs in Cells—Wild-type JunD-FL exhibited a 4-fold stronger transcriptional activity than ∆JunD (Fig. 7A). JNK activation increased both JunD-FL and ∆JunD activity significantly. Curiously, although ∆JunD basal and JNK-activated activities were lower than the corresponding activities for JunD-FL, the fold increase for the JNK-stimulation was higher (3.4-fold for JunD-FL versus 7.5-fold for ∆JunD). This result was somewhat out of line with expectations based on the in vitro data showing ∆JunD to be a less efficient substrate for JNK, suggesting that ∆JunD or JunD-FL may interact with additional factors in the nucleus that modulates their activation state (e.g., the Menin tumor suppressor protein binding and repressing the activity of JunD-FL). Mutation of the JNK docking domain (L57A/L59A) decreased basal activity and had a strong negative effect on JNK activation for both JunD isoforms. These data are consistent with these mutant proteins being incapable of effectively interacting with JNK in the nucleus. Mutation of the serine 90 and serine 100 phosphorylation sites to alanine had a similar effect; both decreased the basal and JNK-stimulated activities. The Ala117 mutants exhibited no decrease in the basal activation level, and a drop in JNK activation of about 40% and little difference was seen between the basal and JNK-activated levels for the triple mutant (S90A/S100A/S117A) compared with the double S90A/S100A.

Altogether these data identify a functional JNK docking domain in both isoforms of JunD. Good agreement was observed between in vitro JNK phosphorylation and JunD-FL binding data. The transcriptional properties of the GAL4-JunD fusion proteins also corresponded well to the kinase and binding data. Although the affinity of the JunD JNK docking domain for JNK is considerably less than that for c-Jun (14–99% relative affinity in the GST pull-down assays, depending on the JunD isoform tested), the positive impact JNK has on the transcriptional activities of JunD-FL and ∆JunD warrant a careful
consideration of JNK-JunD interactions in discerning the biological properties of JunD.

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