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The MEKK1 SWIM domain is a novel substrate receptor for c-Jun ubiquitylation

Michael A. Rieger, **Tyler Duellman**, **Christopher Hooper**, **Magdalene Ameka**, **Joanna C. Bakowska**, and **Bruce D. Cuevas**
Department of Molecular Pharmacology and Therapeutics, Stritch School of Medicine, Loyola University Chicago, Maywood, IL 60153, USA

Abstract

MEKK1 is a mitogen activated protein kinase kinase kinase (MAP3K) that regulates MAPK activation, and is the only known mammalian kinase that is also a ubiquitin ligase. MEKK1 contains a RING domain within its amino-terminal regulatory region, and MEKK1 has been shown to ubiquitylate the AP-1 transcription factor protein c-Jun, but the mechanism by which MEKK1 interacts with c-Jun to induce ubuiquitylation has not been defined. Proximal to RING domain is a SWI2/SNF2 and MuDR (SWIM) domain of undetermined function. In this report, we demonstrate that the MEKK1 SWIM domain, but not the RING domain directly associates with the c-Jun DNA binding domain, and that the SWIM domain is required for MEKK1-dependent c-Jun ubiquitylation. We further show that this MEKK1 SWIM/Jun interaction is specific, as SWIM domains from other proteins failed to bind c-Jun. We reveal that although the Jun and Fos DNA-binding domains are highly conserved, the MEKK1 SWIM domain does not bind Fos. Finally, we identify the sequence unique to Jun proteins required for specific interaction with the MEKK1 SWIM domain. Therefore we propose that the MEKK1 SWIM domain represents a novel substrate-binding domain necessary for direct interaction between c-Jun and MEKK1 that promotes MEKK1-dependent c-Jun ubiquitylation.

Keywords

MEKK1; zinc finger; c-Jun; transcription factor; ubiquitylation; kinase

INTRODUCTION

Ubiquitylation is a key regulatory process that influences the stability of many cellular proteins, and thereby impacts multiple cellular processes [1, 2], including the control of gene expression, proliferation, and viability [3]. Jun family proteins are transcriptional regulators of numerous genes that control multiple homeostatic functions, and the stability of c-Jun is modulated in part by ubiquitin-mediated degradation [4–6]. c-Jun is a component of the dimeric transcription factor AP-1, which consists of Jun family protein (c-Jun, JunB, JunD) homodimers, or Jun heterodimers that include Fos (c-Fos, FosB, Fra-1, Fra-2), ATF, or Maf family proteins [4]. Extensive study of c-Jun function has revealed that it is a key regulator

Address correspondence to Bruce D. Cuevas, PhD, 2160 South First Ave., Maywood, IL, 60153, bcuevas@lumc.edu, Phone: 708-216-6408, FAX: 708-216-6596.

AUTHOR CONTRIBUTION

Michael Rieger, Tyler Duellman, Christopher Hooper, and Magdalene Ameka developed experimental procedures, and designed and performed experiments resulting in the results described in the manuscript. Joanna Bakowska provided technical expertise required for the execution of the experimental procedures. Michael Rieger and Bruce Cuevas conceptualized and developed the study, and Michael Rieger and Bruce Cuevas compiled and wrote the manuscript.

of gene expression that regulates cell proliferation and survival [4, 5, 7]. c-Jun ubiquitylation-associated degradation is mediated by at least four distinct E3 ligases, including Skp1-Cullin-F-box (SCF) ligase complexes (SCFFbw7), the HECT domain-containing ligase AIP4/Itch, and the RING domain-containing ligases hCOP1 and MEKK1 [10–13]. The mechanisms that govern how ubiquitylation complexes specifically interact with Jun proteins are largely unknown.

Protein ubiquitylation is accomplished through the coordinated action of three distinct proteins: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) [14]. E3 ligases consist of proteins that encode either a HECT or a RING domain [14]. RING ubiquitin ligases promote target protein ubiquitylation by binding to both the E2/ubiquitin complex and to the substrate molecule, thereby facilitating transfer of the ubiquitin from the E2 to the substrate [14]. Ubiquitin ligases are far more abundant than either E1 or E2 proteins, and thus substrate specificity is believed to be encoded within individual RING ligases [14, 15]. In contrast, control of substrate specificity of Cullen RING ligases (CRLs) has been shown to reside apart from the actual RING domain-containing protein, within distinct protein components called substrate receptors [16]. Whether this separation of ubiquitin ligase activity from substrate binding specificity is a feature of RING domain-containing proteins other than CRLs has not yet been determined.

MEKK1 is a MAPK kinase kinase (MAP3K) that phosphorylates and activates the dual specificity kinase MKK4, that in turn phosphorylates the MAPK c-Jun amino-terminal kinase (JNK) [18, 19]. Active JNK phosphorylates c-Jun at multiple sites, thereby enhancing Jun transcriptional activity. MEKK1 is the only MAP3K known to exhibit ubiquitin ligase activity [20]. The MEKK1 E3 activity is contained within its RING domain (amino acids 438-490), that is one of two putative zinc-binding domains encoded within the amino-terminal half of the protein [20, 21]. Immediately amino-terminal to the MEKK1 RING domain is a second predicted zinc finger-like sequence (amino acids 298-388) that contains a $CxCx_5CxH$ motif conforming to a SWI2/SNF2 and MuDR (SWIM) domain [21]. SWIM domain-containing proteins have been identified in organisms as diverse as archaea and mammals [21], but the function of this domain is currently unclear. In this report, we identify the MEKK1 SWIM region as a c-Jun-binding domain that functions as a novel ubiquitylation substrate receptor with c-Jun specificity. As we demonstrate that the MEKK1 SWIM domain is necessary and sufficient for Jun association, our data strongly suggest that the SWIM domain provides ubiquitylation target specificity in a manner similar to that of the CRLs.

EXPERIMENTAL

Antibodies and reagents

The antibodies specific for MEKK1 and AP-1 proteins were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal M2 and polyclonal anti-FLAG antibodies were purchased from Sigma (St. Louis, MO), and anti-hemaglutinin (HA, 12CA5) was purchased from Roche Applied Science (Mannheim, Germany). Polyclonal anti-phospho MKK4 and phosho c-Jun antibodies were purchased from Cell Signaling Technology (Danvers, MA). Alexafluor-conjugated secondary antibodies were purchased from Invitrogen (Grand Island, NY). PAR (4-(2-pyridylazo) resorcinol) and MG132 were purchased from Sigma.

Plasmid vectors

The full-length cDNAs encoding wild type murine MEKK1 and mutant MEKK1 (4A-SWIM, SWIM-deleted) were cloned into pCDNA 3.1/myc-His (Invitrogen). The fragments of MEKK1, ZSWIM1, and ZSWIM2 used to create GST fusion proteins were produced by

PCR and cloned into pGEX-5X-3 (GE Life Science). cDNAs encoding murine Jun and Fos, as well as Jun DNA-binding domain mutants R260I, S270A, M263E and A258R and Jun truncation mutants were produced by PCR and cloned into pcDNA3/FLAG, which was a generous gift of Scott Weed (University of West Virginia). ZSWIM1 cDNA was purchased from OpenBiosystems, and MEX/ZSWIM2 cDNA was a generous gift of Gabriel Nunez (University of Michigan).

Cell culture and transfection

The development of fibroblast lines from MEKK1−/− mice has been described previously [24]. Mouse embryo fibroblasts were isolated from E14.5 embryos and were immortalized by continuous passage for 3 months. The MEKK1 add-back fibroblasts were made by stable transfection of immortalized MEKK1−/− fibroblasts using a full-length mouse MEKK1 cDNA, or mutant MEKK1 (C441A, 4A-SWIM) in pCDNA3.1. The 293T cells were purchased from the ATCC. Cells were cultured in DMEM medium (Invitrogen) containing penicillin/streptomycin (1%; Gibco), and 10% (v/v) fetal calf serum (Atlanta Biologicals) at 37°C in a humidified atmosphere. All transfections were conducted using Lipofectamine Plus (Invitrogen) as per manufacturers recommendations.

Immunofluorescence

293T cells stably expressing MEKK1-eGFP were seeded onto glass coverslips. Cells were fixed in methanol-free 4% formaldehyde in phosphate-buffered saline (PBS) for 15 minutes. Following three PBS washes, the cells were permeabilized for 5 minutes with 0.1%Triton X-100 in PBS. After washing, the cells were blocked in 5% goat serum/PBS for one hour at room temperature, then incubated with anti-c-Jun antibodies in block overnight at 4C. After three washes, the coverslips were incubated with Alexa 594-conjugated anti-mouse antibodies (Invitrogen) and Dapi (Molecular Probes) in block for 1 hour at room temperature. Following washing, cells were mounted in 75% glycerol/25% PBS/Tris pH 7.5.

Immunoprecipitation and immunoblotting

Were indicated, cells were treated with 20 μM MG132 for 5 hours in complete media prior to lysis. Cells were lysed in cold lysis buffer (50 mM HEPES pH 7.25, 1% NP-40, 150 mM NaCl, 2 mM EDTA, 50 μM $ZnCl_2$, 50 μM NaH_2PO_4, 50 μM NaF, 1 mM PMSF, 1 mM sodium orthovanadate), and lysates were cleared by centrifugation at 13,000 X g for 10 minutes at 4°C and post-nuclear detergent cell lysates were collected. Equal amounts of each lysate were then incubated with the appropriate antibody for 2 hours at 4°C. Protein A-conjugated agarose beads were then added to the lysates and mixed for 1 hour. The beads were washed three times, then resuspended in sample buffer (125 mM Tris-HCl pH 6.8, 20% glycerol, 4.6% SDS, 0.1% bromophenol blue and 10% 2-mercaptoethanol). Proteins were separated by SDS-PAGE and transferred to Protran nitrocellulose membranes (Whatman). Membranes were blocked in 5% milk (diluted in Tris-buffered saline and 0.1% Tween-20) and incubated with the appropriate antibody at 4°C overnight. HRP-protein A or HRP-sheep anti-mouse IgG was used as secondary reagent. After extensive washing, the targeted proteins were detected by enhanced chemiluminescence (ECL). Where indicated, blots were stripped by treatment with 2% SDS and 100 mM 2-mercaptoethanol in TBS, and then reprobed with desired antibodies and detected by ECL. To assess protein ubiquitylation, the cells were lysed in the presence of 2% SDS and boiled for 10 minutes at 95°C. Lysates were then diluted 15 times (X15) with lysis buffer prior to immunoprecipitation.

Purification of recombinant c-Jun, GST proteins and GST pulldowns

The glutathione S-transferase (GST) fusion proteins were coupled to glutathione sepharose 4B beads (GE Healthcare) and washed extensively in cold PBS. Total cell lysates were prepared from HEK293T cells transiently transfected with FLAG-tagged full-length Jun or Fos proteins, or truncated Jun proteins. GST fusion protein/bead complexes were incubated with 500 μg of HEK293T cell lysate at 4°C for 2 hours. The beads were washed three times with lysis buffer, boiled in sample buffer and separated by SDS-PAGE for immunoblot analysis. Recombinant Jun was produced by transforming cJun-pMAL-c5e into BL21DE3 *E. coli*. The culture was grown to O.D. $A_{600} \approx 0.4$ and expression of MBP-cJun was induced with 0.3 mM IPTG. MBP-cJun was purified on amylose (New England Biolabs) according to the manufacturer's instructions and cleaved with enterokinase (New England Biolabs) to remove the MBP tag. Jun was purified from remaining enterokinase and MBP using Soybean Trypsin Inhibitor agarose (Sigma) or amylose resin.

Extraction of nuclear proteins

Nuclear proteins were extracted as previously described [17]. Briefly, cells were washed in PBS, collected in homogenization buffer (10mM HEPES pH 7.9, 10mM KCl, 1.5mM MgCl2, 0.1mM EGTA, 0.5mM dithiothreitol) using a cell scraper, and the membranes disrupted by passage through a 25-gauge needle. Nuclei were collected by centrifugation, washed twice with homogenization buffer, and resuspended in 50 μl of extraction buffer (10mM HEPES pH 7.90, 0.4M NaCl, 1.5mM MgCl2, 0.1mM EGTA, 0.5mM dithiothreitol), shaken at 4°C for 15 minutes, centrifuged at 4°C for 15 minutes, and supernatant was harvested for immunoblot analysis. The protease inhibitors leupeptin (10 mM), and pepstatin A (10 mM) were included in both the homogenization and extraction buffers.

MTT cell viability assay

Cell viability was assessed by MTT (3,(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide, Sigma) dye conversion at 570 nm as described [25]. Briefly, cells were seeded in 96-well plates (flat bottom, 5000 cells/well). At each time point, 25 μl of MTT (5 mg/ml in PBS) was added to each well. After a 2 hour incubation at 37°C, cells were lysed by addition of 100 μl of 20% SDS in 50% DMF, pH 4.7.

MEKK1 kinase assay

500 μg of total protein from transfected 293T cell lysates was brought to a volume of 350 μl with lysis buffer and rotated end-over-end at 4 °C with 30 μl of Ni-NTA beads (PharMingen). After the 2–4 hour incubation, the beads were washed twice in lysis buffer and once in kinase buffer (20 mM HEPES, pH 7.5, 10 mM MgCl2, 5 mM *p*-nitrophenyl phosphate). The beads were then incubated for 20 min at 30 °C in 50 μl of kinase buffer supplemented with and 1 μg of purified MKK4. The mixture was separated by SDS-PAGE and transferred to nitrocellulose membranes and then immunoblotted with anti-phospho-MKK4 antibodies.

Zinc-binding Assay

This method was adapted from the procedure described by Hunt and colleague [26]. Briefly, $ZnCl_2$ was dissolved in 40 mM HEPES, pH 7 and then mixed with the GST-fusion proteins ($ZnCl_2$ working concentration 15 μM). The mixture was then incubated for 24 hours at 4°C. Afterward, the beads were then washed twice with PBS to remove residual free $ZnCl_2$. The GST-bound proteins were then eluted in (25 mM reduced glutathione, 50 mM Tris pH 8.8, 0.2 M NaCl) for 2–4 hours at 4°C (until absorbance $A_{280} \geq 0.5$, where 1 absorbance unit = 0.5 mg/ml protein). From each sample 25 μg protein was denatured in 4 M guanidine hydrochloride for exactly 3 minutes to release the bound zinc. PAR (4-(2-pyridylazo)

resorcinol, dissolved in water/KOH pH 8.8) was added to each sample (final concentration 100 uM), with free zinc indicated by the change in absorbance (A_{500nm}).

RESULTS

The SWIM domain is necessary for MEKK1 to regulate cell viability

The SWIM domain is encoded within the amino-terminal half of MEKK1 (Figure 1A), proximal to the RING domain. In order to assess the role of the SWIM domain in the regulation of MEKK1-dependent cellular function, we utilized MEKK1-deficient immortalized mouse embryo fibroblasts as recipients of expression vectors that encode either wild type MEKK1 or mutant MEKK1 cDNA. We then used these “add-back” clones that stably expressed different MEKK1 proteins to assess the requirement for distinct MEKK1 domains in cellular function. MEKK1 is a known regulator of apoptosis, thus we examined whether an intact SWIM domain was necessary for MEKK1-dependent regulation of cell viability. By comparing the MTT dye conversion of MEKK1-deficient cells to that of the add-back lines. We observed that the add-back cell line expressing wild type MEKK1 showed a reproducible reduction in MTT dye conversion when compared to MEKK1-deficient cells (Figure 1B). Strikingly, this MEKK1-dependent MTT activity was reversed in both of the add-back lines that expressed mutant MEKK1. Cells expressing mutant MEKK1 with a nonfunctional RING domain (C441A) showed a marked increase in MTT activity consistent with enhanced cell number. Interestingly, add-back cells expressing MEKK1 with mutated SWIM domain (SWIM 4-A) also showed enhanced MTT (Figure 1B). In this SWIM-4A mutant MEKK1, we had replaced the conserved SWIM domain cysteines and histidine with alanines, and our results indicated that these conserved amino acids were required for the observed MEKK1-dependent reduction in MTT activity. Interestingly, trypan dye exclusion was similar between all four lines (data not shown), suggesting that the observed differences in MTT between the lines was due to enhanced cell proliferation in the MEKK1-deficient cells and the cells expressing mutant MEKK1.

Xia and colleagues reported that MEKK1 is a c-Jun E3 ligase that promotes Jun degradation [13], and since c-Jun has been extensively linked to cell proliferation, we investigated whether the observed MEKK1-dependent reduction in MTT was associated with a corresponding reduction in c-Jun expression. Given that the E3-ligase defective MEKK1 mutant markedly enhanced add-back cell viability, we predicted the C441A add-back cells to display a corresponding increase in c-Jun protein relative to the wild type MEKK1 add-back cells. Strikingly, we observed that the fibroblasts expressing the C441A RING mutant MEKK1 exhibit c-Jun protein levels that were markedly increased relative to that of cells expressing wild type MEKK1 (Figure 1C), confirming the importance of MEKK1 ubiquitin ligase activity in control of Jun protein levels. Interestingly, the add-back line that expressed MEKK1 with a mutated SWIM domain motif likewise exhibited increased c-Jun protein (Figure 1C), indicating that the SWIM domain function also regulated Jun protein levels and cell viability.

It has been reported that JNK-dependent phosphorylation targets Jun for ubiquitylation and destruction [11], and so we asked whether the reduced c-Jun protein levels observed in wild type add-back cells were associated with increased JNK-dependent c-Jun phosphorylation. We performed immunoblot analysis on the fibroblast lysates with antibodies specific for Jun proteins phosphorylated at serine 73, which is an activating phospho-acceptor site targeted by JNK. Phosphorylated c-Jun was only detected in the add-back lines and only upon prolonged exposure (Figure 1D), suggesting that the MEKK1 present in cycling add-back cells was sufficient to modestly activate JNK. Importantly, c-Jun phosphorlation at S73 was not associated with reduced Jun expression in the C441A and SWIM-4A add-back cell lines. Overall, our data support the reported role of MEKK1 in c-Jun protein expression. In

addition, our results indicate that the MEKK1 SWIM domain is necessary for the regulation of MEKK1-dependent c-Jun expression, therefore we investigated SWIM domain function in order to elucidate the mechanism by which this occurs.

The SWIM domain is required for MEKK1-dependent Jun ubiquitylation, but not JNK activation

As Nishito and colleagues had recently shown the SWIM domain of the RING domain-containing protein MEX/ZSWIM2 to regulate MEX ubiquitin ligase activity [22], we predicted that the MEKK1 SWIM domain would influence MEKK1-dependent c-Jun ubiquitylation. We used a transfection system to determine whether the SWIM domain was required for Jun ubiquitylation and chose 293T cells as a model, as we have found that these cells do not express detectable levels of endogenous MEKK1. When cells transiently expressing FLAG-tagged c-Jun were treated with the proteasome inhibitor MG132, we observed multiple higher molecular weight forms c-Jun upon immunoblot analysis with anti-FLAG antibodies (Figure 2A), consistent with the presence of multiple forms of ubiquitylated c-Jun. We consistently observed these larger c-Jun forms in MG132-treated cells, even in the absence of exogenous MEKK1, suggesting that one or more endogenous E3 ligases are expressed in 293T cells at a level sufficient to promote some c-Jun ubiquitylation. However, even in the presence of these endogenous E3 ligases, MEKK1 co-expression markedly enhanced the number and intensity of the c-Jun bands, consistent with MEKK1-dependent c-Jun ubiquitylation and similar to the previously reported findings [13]. To confirm that transfected MEKK1 mediates attachment of endogenous ubiquitin to c-Jun, we immunoprecipitated c-Jun and attempted to detect ubiquitylated c-Jun with anti-ubiquitin antibodies. However, we found none of the anti-ubiquitin antibodies used in these experiments to be suitable for detection of endogenous ubiquitin and thus inadequate to clearly differentiate ubiquitylated c-Jun bands from immunoprecipitated protein. To improve detection of ubiquitylated c-Jun, we included co-transfected HA-tagged ubiquitin in our subsequent Jun ubiquitylation experiments (Figure 2C).

We next wanted to determine whether the SWIM domain influenced MEKK1-dependent c-Jun ubiquitylation. In order to examine the requirement for the SWIM domain in Jun ubiquitylation we produced a mutant MEKK1 expression vector in which we removed the sequence encoding the SWIM domain, thereby resulting in expression of an MEKK1 protein without a SWIM domain (amino acids 298-388) that was dubbed SWIM-delete or SD. To assess the impact of SWIM domain removal on MEKK1 kinase activity, we purified His-tagged SD MEKK1 from transfected cells with nickel-charged affinity resin and assayed MEKK1 activity. SD MEKK1 clearly retained the capability to phosphorylate substrate protein MKK4 *in vitro* at a level similar to that of wild type MEKK1 (Figure 2B), and thus we concluded that SD MEKK1 was catalytically intact, and that the SWIM domain was not required for MEKK1-dependent MKK4 phosphorylation. We then co-expressed wild type MEKK1 or SD MEKK1 with HA-tagged ubiquitin and FLAG-tagged c-Jun to determine whether the SD MEKK1 was capable of ubiquitylating c-Jun. Consistent with our *in vitro* kinase assay results, we observed that expression of transfected SD MEKK1 induced activation of JNK pathway signaling resulting in JNK phosphorylation (Figure 2C) similar to that associated with expression of wild type MEKK1. Further, anti-HA immunoblots of immunoprecipitated FLAG-c-Jun revealed that c-Jun co-expressed with wild type MEKK1 was strongly ubiquitylated (Figure 2C, top panel). In striking contrast, we observed a dramatic reduction in c-Jun ubiquitylation when c-Jun was co-expressed with the SD mutant MEKK1 (Fig. 2C), indicating that SD MEKK1 is less effective than wild type MEKK1 at inducing c-Jun ubiquitylation. It is important to note that, although these experiments with transfected HA-ubiquitin did not include MG132 that would inhibit proteasomal degradation of c-Jun, we did not observe a loss of transiently expressed c-Jun protein in 293T cells that

mirrored our data with endogenous c-Jun stably transfected fibroblasts (Figure 1C). One possible explanation for this observation is that the transiently expressed c-Jun is produced at higher levels than in the fibroblasts that more than compensates for c-Jun protein lost to proteasomal degradation, thereby masking ubiquitin-mediated c-Jun destruction. Alternatively, high expression of exogenous ubiquitin may alter the kinetics of proteasome-mediated protein removal. Regardless, our data confirm that wild type MEKK1 promotes c-Jun ubiquitylation and strongly suggest that the SWIM domain is necessary for efficient ubiquitylation of c-Jun by MEKK1.

c-Jun is a transcription factor that binds DNA and thus is localized in the nucleus. As MEKK1 is a large kinase with established cytoplasmic functions, we asked whether MEKK1 could localize to the nucleus in 293T cells. We observed that a green fluorescent protein-linked MEKK1 protein localized to the cell nucleus in a portion of transfected cells, thereby colocalizing with nuclear c-Jun (Supplementary Figure 1A). To determine whether mutant MEKK1 could likewise localize to the cell nucleus, we again expressed SD MEKK1 and the RING domain mutant MEKK1 C441A in cells and performed cell fractionation to examine the distribution of MEKK1 proteins to the cytoplasmic and nuclear compartments. Our analysis confirmed that all MEKK1 proteins tested were distributed similarly to both the cytoplasm and the nucleus (Supplementary Figure 1B). Taken together, our results strongly suggest that loss of MEKK1-dependent c-Jun ubiquitylation associated with SD MEKK1 expression was due to ineffective c-Jun ubuiquitylation and not caused by reduced kinase activity or altered localization of mutant MEKK1 proteins.

MEKK1 SWIM directly associates with Jun

To determine how the MEKK1 SWIM domain regulates c-Jun ubiquitylation, we asked whether c-Jun physically associated with the SWIM domain. We developed and purified GST fusion proteins containing either SWIM or RING domains, and then mixed these recominant proteins with FLAG epitope-tagged c-Jun from transfected cell lysates, separated the associated proteins by SDS-PAGE and performed immunoblot analysis to detect c-Jun association. We found that the SWIM domain alone was sufficient to pull down c-Jun, whereas RING did not detectably associate with c-Jun (Fig. 3A).

The MEKK1 SWIM domain includes a conserved $CXCX_{(n)}CXH$ motif that was identified by alignment with other SWIM domain-containing proteins (Figure 1A) [21]. This SWIM motif is similar to the signature motif found in C3H zinc finger proteins ($CX_{(8)}CX_{(5)}CX_{(3)}H$) [27, 28], and as zinc fingers frequently mediate protein-to-protein interaction, we asked whether the MEKK1 SWIM was a zinc-binding domain. Utilizing a colorimetric assay to detect free zinc eluted from purified proteins, we determined the relative zinc-chelating activity of the SWIM and RING domains (Supplementary Figure 2) and discovered that the SWIM zinc-binding activity is similar to that of the RING domain. Importantly, the capacity of SWIM to bind zinc was abolished when the SWIM motif amino acids were mutated to alanines (CCCHA), indicating that these conserved residues are required by the SWIM domain to chelate zinc from solution. We next wanted to determine whether the MEKK1 SWIM domain could directly bind c-Jun, and so we utilized an *in vitro* binding approach in which purified recombinant GST-linked SWIM domain is combined with purified recombinant c-Jun, and then associated c-Jun would be detected by immunoblot (Fig. 3B). We observed that purified c-Jun was pulled down by GST-SWIM but not GST alone, clearly indicating that the MEKK1 SWIM could directly bind c-Jun.

As with the MEKK1 SWIM domain, the binding specificities of the SWIM domains encoded within other proteins have not yet been defined. To determine whether the SWIM domains of other proteins function as c-Jun-binding domains, we purified GST-linked recombinant SWIM domains from human proteins ZSWIM1 and MEX/ZSWIM2, and then

combined these domains with recombinant c-Jun. In contrast to the MEKK1 SWIM domain, the SWIM domains of ZSWIM1 and MEX/ZSWIM2 failed to associate with c-Jun in vitro (Figure 3C), indicating that the ZSWIM domains did not directly bind c-Jun, and suggesting that these ZSWIM peptides were not c-Jun-binding domains.

Finally, to assess whether the MEKK1 SWIM domain can form a complex with c-Jun in cells, we immunoprecipitated MEKK1 from transfected cell lysates and determined whether c-Jun was co-immunoprecipitated with MEKK1. We found that c-Jun did indeed co-immunoprecipitate with full-length MEKK1 (Figure 3D). We then compared the c-Jun binding capacity of wild type MEKK1 to that of MEKK1 mutant SWIM-4A to determine whether the SWIM domain was required for c-Jun complex formation with full length protein. Consistent with our findings in the GST system, c-Jun co-immunoprecipitated with the wild type full length MEKK1, but failed to associate with the SWIM-4A mutant MEKK1 (Fig. 3D), indicating that the conserved SWIM motif residues are required for MEKK1 association with c-Jun. Taken together, our data indicate that the MEKK1 SWIM domain is a c-Jun-binding domain that is necessary for efficient MEKK1-dependent c-Jun ubiquitylation. Our results also suggest that forming a complex with c-Jun is not a general function of SWIM domain-containing proteins, but rather that physical association with c-Jun is specific to the MEKK1 SWIM domain.

MEKK1 SWIM domain specifically associates with Jun DNA-binding domain

To identify the region of c-Jun required for direct interaction with the MEKK1 SWIM domain, we developed a series of FLAG epitope-tagged c-Jun carboxyl-terminal truncation mutants (Figure 4A) and assessed the ability of each mutant c-Jun protein to associate with recombinant SWIM domain. Immunoblot analysis GST pulldowns of transfected cell lysates clearly indicated that the c-Jun carboxyl terminus and leucine zipper regions were dispensable for association with the MEKK1 SWIM domain (Fig. 4B). As the leucine zipper region of Jun proteins is necessary for dimer formation [29, 30], these data lead us to conclude that the MEKK1 SWIM domain can bind c-Jun monomers and that AP-1 dimerization is not required for MEKK1/c-Jun complex formation. However, elimination of the c-Jun DNA-binding domain (DBD, amino acids 255-276) completely blocked association between c-Jun and SWIM (Figure 4B), indicating that the Jun DNA-binding domain is required for association of c-Jun with the MEKK1 SWIM domain.

Since the DBD of Jun and Fos family proteins are highly similar [29, 30] (Fig. 5B), we asked whether the MEKK1 SWIM domain also could associate with Fos proteins. We utilized GST-SWIM fusion proteins to pull down FLAG-tagged Jun and Fos family proteins from transfected cell lysates and then assessed complex formation by immunoblot analysis. Surprisingly, we discovered that recombinant SWIM domain was capable of binding c-Jun, JunB and JunD proteins, but did not associate with either Fos or Fra2 proteins (Fig. 5A). We were able to detect the Fos proteins only upon prolonged exposure of the SWIM pulldown immunoblots (data not shown), suggesting that Fos protein association with SWIM domain is indirect and mediated only through association with endogenous Jun.

To understand why Fos proteins failed to associate with the MEKK1 SWIM domain, we compared the amino acid sequence of the DBD of Jun and Fos proteins, and found that while ten amino acid residues are completely conserved in both Jun and Fos proteins, the non-conserved amino acids were distinctive between the two protein families. For example, a basic arginine residue is encoded in c-Jun at position 260, and this is conserved in all Jun proteins at that position, but the corresponding residue in Fos proteins is a hydrophobic isoleucine residue (Figure 5B). This observation suggested to us that one or more of the amino acid residues found in Jun, but not Fos proteins are required for SWIM complex formation. To identify the required Jun DBD residues, we replaced Jun amino acids with the

analogous amino acids present in Fos proteins and asked whether the mutant Jun proteins retained the capacity to associate with the MEKK1 SWIM domain. Our immmunoblot analysis revealed that replacement of arginine 260 in the Jun DBD with an isoleucine blocked association with the SWIM domain (Figures 5C, D). Replacement of serine 270 in Jun proteins with the analogous Fos alanine also inhibited association with SWIM to a lesser degree, but replacing Jun alanine 258 and methionine 263 with charged amino acids found in Fos proteins did not inhibit association with SWIM. These data indicate that c-Jun association with the MEKK1 SWIM domain is mediated by interactions between the SWIM domain and an amino acid that is conserved in the Jun DBD, but not the Fos DBD.

DISCUSSION

In this study, we define the SWIM domain as a novel substrate-binding domain for MEKK1 ubiquitin ligase activity that specifically binds to c-Jun to promote c-Jun ubiquitylation. As a consequence of these studies we have further delineated the regulatory mechanisms that control AP-1 activity. Further, our work is the first to define a specific function for a SWIM domain, and elucidates the mechanism by which MEKK1 captures a ubiquitylation substrate protein. Identifying proteins targeted by MEKK1 or other E3 ligases has proven to be challenging due to the limited current understanding of the mechanisms that determine RING E3 ligase substrate specificity. While MEKK1 has been reported to ubiquitylate c-Jun and ERK1 [13, 22], the mechanisms that control complex formation between these proteins and MEKK1 were not addressed. Our results indicate that the association between MEKK1 and c-Jun necessary for MEKK1-dependent c-Jun ubiquitylation is mediated by the SWIM domain and is independent of the RING domain. We further conclude that the SWIM domain functions as an ubiquitin ligase substrate receptor module similar to the substrate receptor proteins that interact with Cullin proteins to form CRL ubiquitylation complexes. CRLs utilize a modular system wherein substrate specificity is determined by a protein linked to the RING domain-containing protein via a Cullin protein scaffold. This analogy is imperfect, however, as the CRLs may complex with multiple substrate receptor components with differing substrate specificities, whereas our model of SWIM domain-dependent ubiquitylation limits MEKK1 to a single substrate receptor that specifically binds Jun proteins. The possibility that other SWIM domain-containing proteins function as independent E3 substrate binding subunits is an intriguing one that will require further investigation to establish. We did not observe MEKK1 to associate with other SWIM domain-containing proteins.

To determine whether Jun complex formation is a general characteristic of SWIM domains, we examined the Jun-binding capacity of SWIM domains from two other proteins that are structurally and functionally distinct from MEKK1. In contrast to the SWIM domains of ZSWIM1 and MEX/ZSWIM2, we found that the MEKK1 SWIM domain alone bound c-Jun. We did not identify the specificity determinants that distinguished the MEKK1 SWIM domain from similar domains in ZSWIM1 and MEX/ZSWIM2 as part of this study. However, a comparison of the primary sequence of the SWIM domains within the three proteins reveals that, apart from the conserved cysteine and histidine motif residues, the sequences are heterogeneous (Supplementary Figure 3, UniProt accession numbers for ZSWIM1, ZSWIM2 and MEKK1 are Q9CWV7, Q0VB10 and F8VQ72 respectively), which suggests that SWIM domain binding specificity is determined by interactions unique to a given sequence. In addition, the spacing of the cysteine residues within the ZSWIM proteins is distinct from that of MEKK1, and thus we cannot rule out the possibility that the separation of the cysteine residues within the SWIM motif may influence binding specificity.

In our previous MEKK1 localization studies [35], we observed that a subset of MEKK1 is co-localized with actin stress fibers in the cytoplasm, and we demonstrated that cytoskeletal MEKK1 regulates cell adhesion. The results of our current study reveal that MEKK1 localizes to more than one cellular compartment to regulate distinct functions. We would postulate that signaling events that promote MEKK1 nuclear localization reduce c-Jun protein stability, whereas factors that retain MEKK1 in the cytoplasm would enhance c-Jun stability. The mechanism by which MEKK1 moves to the nucleus is not clear at this time. MEKK1 does not contain a consensus nuclear localization sequence (NLS), thus either MEKK1 contains a non-consensus NLS, or it associates with other proteins that can transport MEKK1 to the nucleus. Candidate proteins for this “MEKK1 carrier” protein would include signaling effectors that are induced to move from the cytoplasm to the nucleus in response to an environmental stimulus. Interestingly, Stat family transcription factors fit this general description, and MEKK1 has been reported to associate with Stat3 in response to EGF [32, 33]. Our future studies we will be designed to define the mechanism by which MEKK1 localizes to the nucleus.

In summary, our study shows that the specific and direct association between Jun and the MEKK1 SWIM domain produces a novel regulatory protein complex that modulates c-Jun availability by SWIM domain-dependent c-Jun ubiquitylation. We define for the first time a function for a SWIM domain, and identify the Jun components necessary for interaction with MEKK1. Finally, we propose a model in which the MEKK1 SWIM domain functions as a E3 ligase substrate binding domain analogous to substrate receptors of the CRL ubiquitylation complexes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

AP-1	activator protein-1
DBD	DNA-binding domain
ERK1	extracellular signal-regulated kinase-1
JNK	c-Jun amino terminal kinase
MAPK	mitogen activated protein kinase
MAP3K	MAPK kinase kinase
MEKK1	MEK kinase 1
MKK4	MAPK kinase 4
RING	really interesting new gene
SWIM	SWI2/SNF2 and MuDR

References

1. Varshavsky A. Regulated protein degradation. Trends Biochem Sci. 2005; 30:283–286. [PubMed: 15950869]
2. Goldberg AL. Functions of the proteasome: from protein degradation and immune surveillance to cancer therapy. Biochem Soc Trans. 2007; 35:12–17. [PubMed: 17212580]
3. Schwartz AL, Ciechanover A. Targeting proteins for destruction by the ubiquitin system: implications for human pathobiology. Annu Rev Pharmacol Toxicol. 2009; 49:73–96. [PubMed: 18834306]
4. Shaulian E, Karin M. AP-1 as a regulator of cell life and death. Nat Cell Biol. 2002; 4:E131–136. [PubMed: 11988758]
5. Shaulian E, Karin M. AP-1 in cell proliferation and survival. Oncogene. 2001; 20:2390–2400. [PubMed: 11402335]
6. Anzi S, Finkin S, Shaulian E. Transcriptional repression of c-Jun's E3 ubiquitin ligases contributes to c-Jun induction by UV. Cell Signal. 2008; 20:862–871. [PubMed: 18295447]
7. Angel P, Karin M. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim Biophys Acta. 1991; 1072:129–157. [PubMed: 1751545]
8. Rodig SJ, Ouyang J, Juszczynski P, Currie T, Law K, Neuberg DS, Rabinovich GA, Shipp MA, Kutok JL. AP1-dependent galectin-1 expression delineates classical hodgkin and anaplastic large cell lymphomas from other lymphoid malignancies with shared molecular features. Clin Cancer Res. 2008; 14:3338–3344. [PubMed: 18519761]
9. Mathas S, Hinz M, Anagnostopoulos I, Krappmann D, Lietz A, Jundt F, Bommert K, Mechta-Grigoriou F, Stein H, Dorken B, Scheidereit C. Aberrantly expressed c-Jun and JunB are a hallmark of Hodgkin lymphoma cells, stimulate proliferation and synergize with NF-kappa B. EMBO J. 2002; 21:4104–4113. [PubMed: 12145210]
10. Nateri AS, Riera-Sans L, Da Costa C, Behrens A. The ubiquitin ligase SCFFbw7 antagonizes apoptotic JNK signaling. Science. 2004; 303:1374–1378. [PubMed: 14739463]
11. Gao M, Labuda T, Xia Y, Gallagher E, Fang D, Liu YC, Karin M. Jun turnover is controlled through JNK-dependent phosphorylation of the E3 ligase Itch. Science. 2004; 306:271–275. [PubMed: 15358865]
12. Wertz IE, O'Rourke KM, Zhang Z, Dornan D, Arnott D, Deshaies RJ, Dixit VM. Human De-etiolated-1 regulates c-Jun by assembling a CUL4A ubiquitin ligase. Science. 2004; 303:1371–1374. [PubMed: 14739464]
13. Xia Y, Wang J, Xu S, Johnson GL, Hunter T, Lu Z. MEKK1 mediates the ubiquitination and degradation of c-Jun in response to osmotic stress. Mol Cell Biol. 2007; 27:510–517. [PubMed: 17101801]
14. Deshaies RJ, Joazeiro CA. RING domain E3 ubiquitin ligases. Annu Rev Biochem. 2009; 78:399–434. [PubMed: 19489725]
15. van Wijk SJ, de Vries SJ, Kemmeren P, Huang A, Boelens R, Bonvin AM, Timmers HT. A comprehensive framework of E2-RING E3 interactions of the human ubiquitin-proteasome system. Mol Syst Biol. 2009; 5:295. [PubMed: 19690564]
16. Petroski MD, Deshaies RJ. Function and regulation of cullin-RING ubiquitin ligases. Nat Rev Mol Cell Biol. 2005; 6:9–20. [PubMed: 15688063]
17. Cuevas BD, Uhlik MT, Garrington TP, Johnson GL. MEKK1 regulates the AP-1 dimer repertoire via control of JunB transcription and Fra-2 protein stability. Oncogene. 2005; 24:801–809. [PubMed: 15558021]
18. Yujiri T, Sather S, Fanger GR, Johnson GL. Role of MEKK1 in cell survival and activation of JNK and ERK pathways defined by targeted gene disruption. Science. 1998; 282:1911–1914. [PubMed: 9836645]
19. Cuevas BD, Abell AN, Johnson GL. Role of mitogen-activated protein kinase kinase kinases in signal integration. Oncogene. 2007; 26:3159–3171. [PubMed: 17496913]
20. Witowsky JA, Johnson GL. Ubiquitylation of MEKK1 inhibits its phosphorylation of MKK1 and MKK4 and activation of the ERK1/2 and JNK pathways. J Biol Chem. 2003; 278:1403–1406. [PubMed: 12456688]

21. Makarova KS, Aravind L, Koonin EV. SWIM, a novel Zn-chelating domain present in bacteria, archaea and eukaryotes. Trends Biochem Sci. 2002; 27:384–386. [PubMed: 12151216]
22. Lu Z, Xu S, Joazeiro C, Cobb MH, Hunter T. The PHD domain of MEKK1 acts as an E3 ubiquitin ligase and mediates ubiquitination and degradation of ERK1/2. Mol Cell. 2002; 9:945–956. [PubMed: 12049732]
23. Nishito Y, Hasegawa M, Inohara N, Nunez G. MEX is a testis-specific E3 ubiquitin ligase that promotes death receptor-induced apoptosis. Biochem J. 2006; 396:411–417. [PubMed: 16522193]
24. Yujiri T, Ware M, Widmann C, Oyer R, Russell D, Chan E, Zaitsu Y, Clarke P, Tyler K, Oka Y, Fanger GR, Henson P, Johnson GL. MEK kinase 1 gene disruption alters cell migration and c-Jun NH2-terminal kinase regulation but does not cause a measurable defect in NF-kappa B activation. Proc Natl Acad Sci U S A. 2000; 97:7272–7277. [PubMed: 10852963]
25. Hansen MB, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J Immunol Methods. 1989; 119:203–210. [PubMed: 2470825]
26. Hunt JB, Neece SH, Ginsburg A. The use of 4-(2-pyridylazo)resorcinol in studies of zinc release from Escherichia coli aspartate transcarbamoylase. Anal Biochem. 1985; 146:150–157. [PubMed: 3887984]
27. Laity JH, Lee BM, Wright PE. Zinc finger proteins: new insights into structural and functional diversity. Curr Opin Struct Biol. 2001; 11:39–46. [PubMed: 11179890]
28. Gamsjaeger R, Liew CK, Loughlin FE, Crossley M, Mackay JP. Sticky fingers: zinc-fingers as protein-recognition motifs. Trends Biochem Sci. 2007; 32:63–70. [PubMed: 17210253]
29. Junius FK, O'Donoghue SI, Nilges M, Weiss AS, King GF. High resolution NMR solution structure of the leucine zipper domain of the c-Jun homodimer. J Biol Chem. 1996; 271:13663–13667. [PubMed: 8662824]
30. Chen L, Glover JN, Hogan PG, Rao A, Harrison SC. Structure of the DNA-binding domains from NFAT, Fos and Jun bound specifically to DNA. Nature. 1998; 392:42–48. [PubMed: 9510247]
31. Schonthaler HB, Guinea-Viniegra J, Wagner EF. Targeting inflammation by modulating the Jun/AP-1 pathway. Ann Rheum Dis. 70(Suppl 1):i109–112. [PubMed: 21339212]
32. Lim CP, Cao X. Regulation of Stat3 activation by MEK kinase 1. J Biol Chem. 2001; 276:21004–21011. [PubMed: 11278353]
33. Nakamura Y, Yujiri T, Nawata R, Tagami K, Tanizawa Y. MEK kinase 1 is essential for Bcr-Abl-induced STAT3 and self-renewal activity in embryonic stem cells. Oncogene. 2005; 24:7592–7598. [PubMed: 16044153]
34. Zhu P, Baek SH, Bourk EM, Ohgi KA, Garcia-Bassets I, Sanjo H, Akira S, Kotol PF, Glass CK, Rosenfeld MG, Rose DW. Macrophage/cancer cell interactions mediate hormone resistance by a nuclear receptor derepression pathway. Cell. 2006; 124:615–629. [PubMed: 16469706]
35. Cuevas BD, Abell AN, Witowsky JA, Yujiri T, Johnson NL, Kesavan K, Ware M, Jones PL, Weed SA, DeBiasi RL, Oka Y, Tyler KL, Johnson GL. MEKK1 regulates calpain-dependent proteolysis of focal adhesion proteins for rear-end detachment of migrating fibroblasts. EMBO Journal. 2003; 22:3346–3355. [PubMed: 12839996]

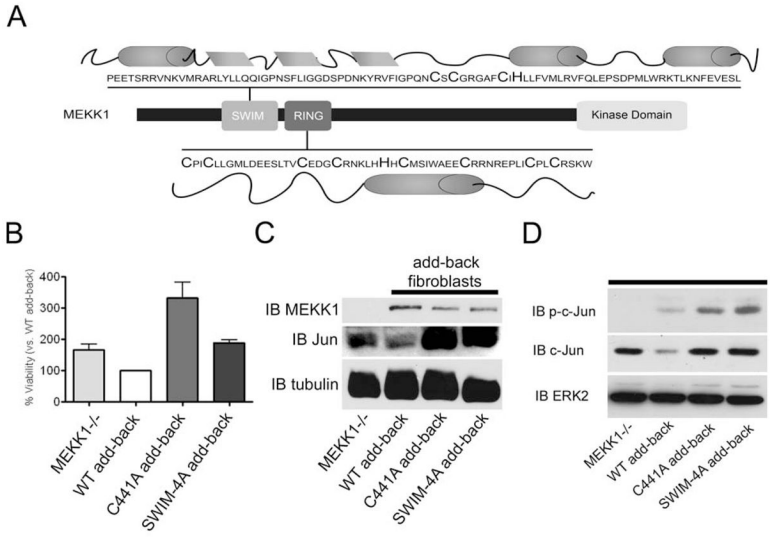

Figure 1. The MEKK1 SWIM domain mutation enhances cell viability and c-Jun expression

(**A**) Display of the sequences included in the MEKK1 SWIM and RING domains, including a graphical representation of the predicted secondary structures of these domains based on the NNPREDICT algorithm. The conserved cysteine residues in both domains are enlarged. (**B and C**) The functional consequence of MEKK1 expression was evaluated in immortalized MEKK1-deficient fibroblasts that stably express either wild type or mutant MEKK1 (add-back cell lines). (**B**) Mean relative fibroblast cell line viability as assessed by MTT is represented. Each bar represents the mean of three independent experiments. *$p<0.05$ (**C**) c-Jun expression is assessed by immunoblot (IB) analysis of add-back cell lysates. The upper panel is an anti-MEKK1 immunoblot showing add-back expression. The middle panel displays the corresponding c-Jun expression in each cell line, and the lower panel shows an anti-tubulin immunoblot to demonstrate equal loading of lysate proteins. (**D**) c-Jun phosphorylation in fibroblast cell lines using antibodies raised to phosphorylated serine residue 73. Displayed blot is representative of at least three experiments.

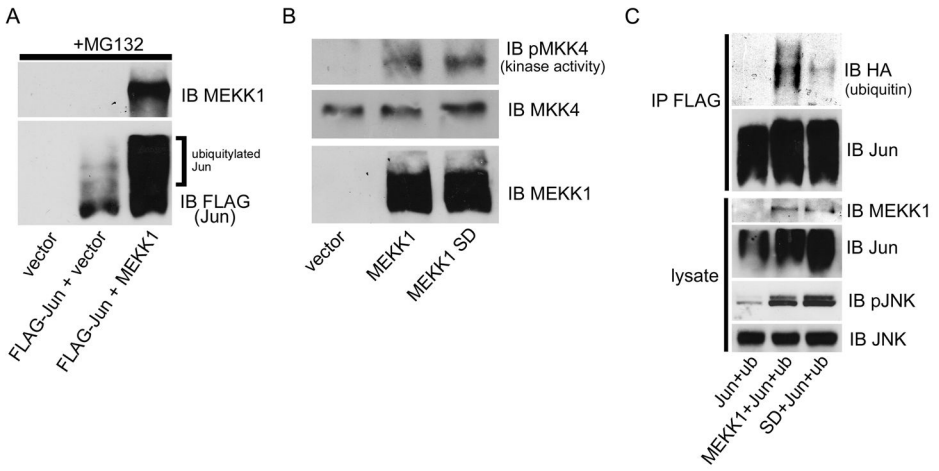

Figure 2. The SWIM domain is required for c-Jun ubiquitylation, but not MKK4 phosphorylation

(A) MEKK1 expression enhances prevalence of higher molecular weight c-Jun protein in the presence of proteasome inhibitor MG132. (**B**) MEKK1 *in vitro* kinase activity is assessed by immunoblot analysis detecting phosphorylated unactive MKK4 (upper panel). Middle panel shows total MKK4 from each reaction, and the lower panel shows immunoblot of purified wild type or SD MEKK1. (**C**) A comparison of ubiquitylation activity of MEKK1 mutant SWIM-deleted to that of wild type MEKK1. Transfected FLAG-c-Jun was co-expressed with HA –tagged ubiquitin and wild type or mutant MEKK1, and ubiquitylation of immunoprecipitated (IP) c-Jun was detected by anti-HA immunoblot (top panel). Lysates (lower panels) from transfected 293T cell lysates display loading and associated level of phosphorylated JNK in each lane. Results are representative of three or more independent experiments.

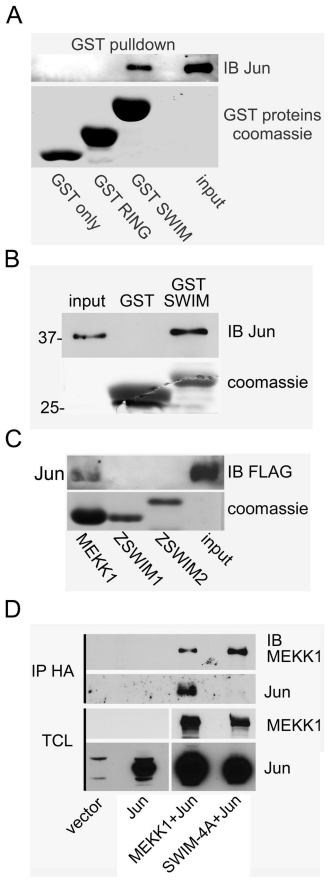

Figure 3. MEKK1 SWIM domain directly and specifically binds Jun

(**A**) GST-SWIM, but not GST-RING forms a complex with c-Jun from 293T cell lysates. In (**A**), (**B**) and (**C**), the upper panels are anti-c-Jun immunoblots, and the lower panels display coomassie stained gel to show the relative amounts of GST fusion proteins. (**B**) GST-SWIM directly binds recombinant c-Jun *in vitro*, but GST alone does not bind c-Jun. (**C**) The MEKK1 SWIM domain binds to c-Jun from 293T cell lysates, but the SWIM domains from proteins ZSWIM1 and ZSWIM2 do not bind c-Jun. Results are representative of at least three independent experiments. (**D**) Immunoblot analysis of c-Jun co-immunoprecipitation with HA-tagged full-length MEKK1. SWIM-4A is a full length MEKK1 in which SWIM signature motif cysteines and histidine have been mutated to alanines. Upper two panels display immunoblots of immunoprecipitated proteins with anti MEKK1 and anti Jun antibodies. The lower two panels are immunoblots of total cell lysates to indicate protein expression and loading. Results are representative of at least three independent experiments.

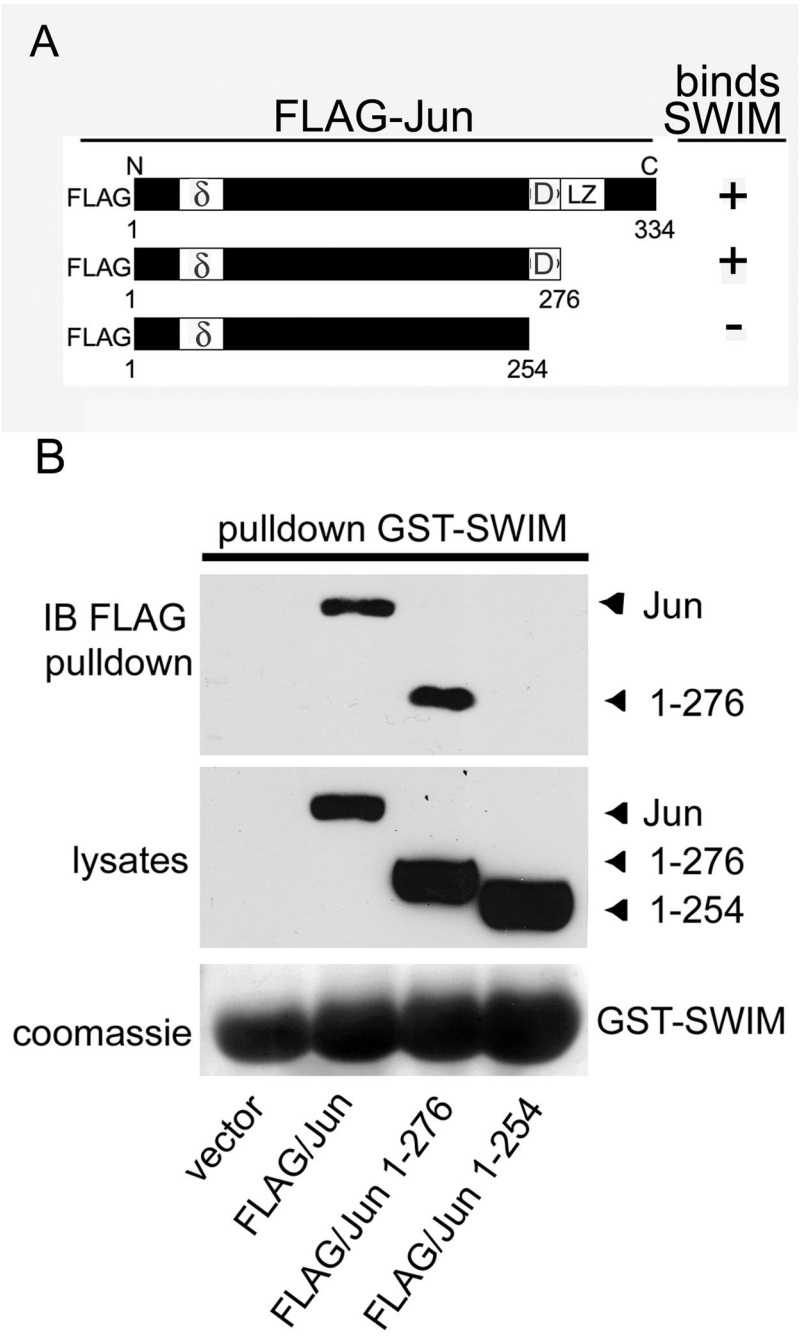

Figure 4. The Jun DNA-binding domain associates with the MEKK1 SWIM domain

(**A**) A graphical representation of the FLAG-tagged full length c-Jun and Jun truncation mutant proteins used in the pulldown experiments. (**B**) GST-SWIM domain fusion protein pulldown of full length c-Jun and truncated c-Jun mutants. Results are representative of at least three independent experiments. δ = delta domain, D = DNA-binding domain, LZ = leucine zipper domain

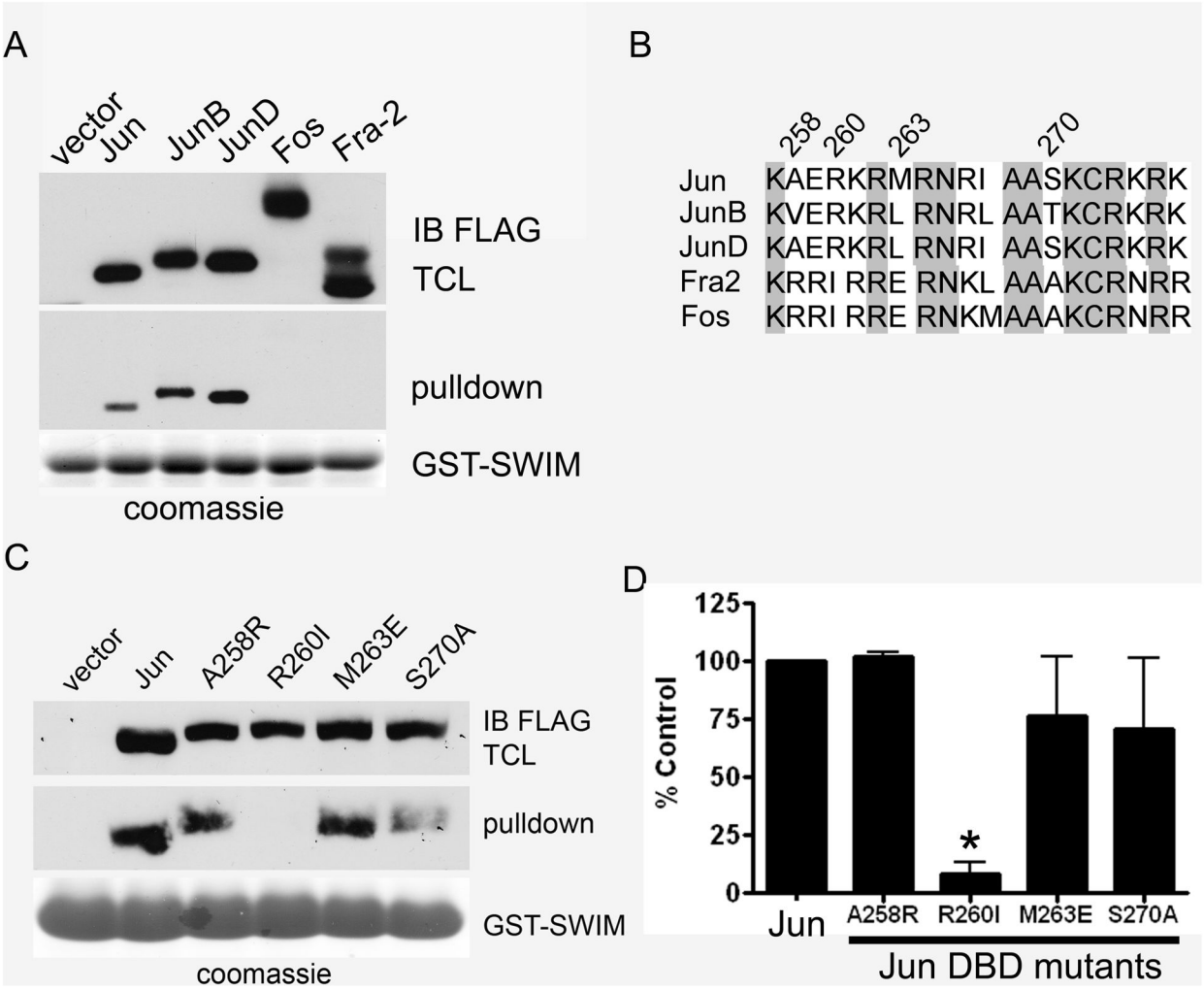

Figure 5. The arginine necessary for SWIM association is conserved in Jun but not Fos proteins

(**A**) GST-SWIM fusion proteins were used to pull down FLAG-tagged AP-1 proteins. Upper panel displays anti-FLAG immunoblot of transfected cell lysates. The middle panel shows anti-FLAG immunoblot to reveal proteins that formed complexes with GST-SWIM. The lower panel is a coomassie-stained gel that includes the GST-SWIM fusion protein to indicate the relative fusion protein levels used in each sample. (**B**) A sequence alignment of the Jun and Fos protein DNA-binding domains. The conserved residues are indicated in gray, and the numbered residues were selected for mutagenesis in c-Jun. UniProt accession numbers for the sequences are as follows: P05627 (c-Jun), P09450 (JunB), P15066 (JunD), P01101 (c-Fos), and P47930 (Fra2). (**C**) GST-SWIM pulldown assay using c-Jun and mutant c-Jun proteins from transfected 293T cells. The upper panel shows an anti-FLAG immunoblot of the total cell lysates to confirm expression of the transfected Jun proteins. The middle panel is an anti-FLAG immunoblot showing the Jun proteins pulled down by the GST-SWIM, and the lower panel shows a coomassie stain of the gel indicating fusion protein loading. (**D**) Graphical summary of mutant c-Jun pulldown results. Densitometry of pulldown was normalized to respective total cell lysate immunoblot and analyzed by student's T test for statistical significance. *p<0.05. Results are representative of at least three independent experiments.