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A Novel Specific Role for IκB Kinase Complex-associated Protein in Cytosolic Stress Signaling*

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We demonstrate here a novel role for the IκB kinase complex-associated protein (IKAP) in the regulation of activation of the mammalian stress response via the c-Jun N-terminal kinase (JNK)-signaling pathway. We cloned IKAP as a JNK-associating protein using the Ras recruitment yeast two-hybrid system. IKAP efficiently and specifically enhanced JNK activation induced by ectopic expression of MEKK1 and ASK1, upstream activators of JNK. Importantly, IKAP also enhanced JNK activation induced by ultraviolet light irradiation as well as treatments with tumor necrosis factor or epidermal growth factor. The JNK association site in IKAP was mapped to the C-terminal part of IKAP. Interestingly, this region is deleted from IKAP expressed in the autonomous nervous system of the patients affected by familial dysautonomia. Ectopic expression of this C-terminal fragment of IKAP was sufficient to support JNK activation. Taken together, our data demonstrate a novel role for IKAP in the regulation of the JNK-mediated stress signaling. Additionally, our results point to a role of JNK signaling in familial dysautonomia and, thus, further support the involvement of JNK signaling in the development, survival, and degeneration of the sensory and autonomic nervous system.

IKAP[1] was originally purified as a 150-kDa-protein component of the large interleukin 1-inducible IKK complex containing NIK, IKKα, IKKβ, and NF-κB/relA and suggested to be a scaffold protein involved in the assembly of the IKK signalosome (1). A later report by Krappmann *et al.* (2) cast doubt on the role of IKAP in the NF-κB pathway. Using a gel filtration approach, these authors were unable to detect IKAP in the fractions containing IKK complexes.

Instead, it was suggested that IKAP is involved in general RNA polymerase II transcription elongation, since the *Saccharomyces cerevisiae* protein Elp1/IKI3, which shares 29% identity with IKAP, co-purifies with a protein complex, termed elongator, containing histone acetylase activity and presumably involved in transcriptional elongation (3). Recently, the orthologous human elongator has been purified as well, and indeed, it contains IKAP (4). IKAP itself does not possess any histone acetylase activity. Thus, its role in transcriptional elongation may be to support the elongator complex assembly (3, 4).

Mutations in the *IKBKAP* gene encoding IKAP have been shown to be responsible for the autosomal recessive disease familial dysautonomia (5, 6). The predominant mutation confers tissue-specific expression of a short version of IKAP truncated at amino acid 714, and homozygousity for the mutation confers poor development, progressive degeneration of the sensory and autonomic nervous system, and early death. Additionally, specific point mutations in the coding region of the *IKBKAP* gene are reported to increase the risk of bronchial asthma in children (7). How *IKBKAP* mutations cause or predispose to these diseases are yet unknown.

Activation of JNK has been shown to be involved in development, proliferation, inflammation, and apoptosis, and JNK is the key protein in addition to the related p38 kinase responsible for the rapid transmission of the extracellular stress signals in mammalian cells (8, 9). The JNK family consists of three different isoforms, JNK1, JNK2, and JNK3, encoded by three closely related genes that can give rise to 55- and 45-kDa protein products (10, 11). JNK1 and JNK2 are ubiquitously expressed, whereas the expression of JNK3 is restricted to brain and testis (11).

Recent studies on mice harboring inactivated JNK1, -2, or -3 genes have demonstrated their importance in T-cell differentiation and/or proliferation as well as in the development of the nervous system (for review, see Davis (12)). Inactivation of the JNK3 gene in mice leads to deficiency in the kainic acid-induced excitoxicity, resulting in decreased apoptosis in the hip-

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[1] The abbreviations used are: IKAP, IκB kinase complex-associated protein; IKK, IκB kinase; NIK, NCK-interacting kinase; JIP, JNK-interacting protein; FD, familial dysautonomia; JNK, c-Jun N-terminal kinase; JNKK, JNK kinase; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPK kinase kinase kinase; MEKK, MEK (MAPK/extracellular signal-regulated kinase kinase) kinase; ASK, apoptosis signaling kinase; JSAP, JNK/SAPK-associated protein; SAPK, stress-activated protein kinase; RRS, Ras recruitment system; TNF, tumor necrosis factor; HA, hemagglutinin; GST, glutathione *S*-transferase; MLK, mixed lineage kinase; DLK, dual leucine zipper kinase; TAK, tumor growth factor β-activated kinase.

pocampus of the JNK3 knockout mice (13). This is probably due to impaired c-Jun phosphorylation since the Jun-ala/ala knock-in mice, in which the major JNK phosphorylation sites (serines 63 and 73) are replaced with alanine residues, also exhibit impaired response to kainic acid (14). JNK1 and -2 double knockout mice die around embryonic day 10, apparently due to a defect in neural tube closure that involves decreased apoptosis in the embryonic hindbrain. Interestingly, in the embryonic forebrain lack of both JNK1 and JNK2 causes the opposite phenotype because they are necessary for the survival of the developing cortical neurons (15, 16).

Since the cloning of the human and rat JNK isoforms (11, 17–20), also known as the stress activated protein kinases (21), a vast number of studies have been carried out to elucidate how extracellular signals are mediated to this kinase family. This work has revealed the existence of a complex network of kinases and other regulatory proteins responsible for JNK activation. It is now well established that JNK, which belongs to the group of mitogen-activated protein kinase (MAPK)-signaling enzymes, is activated within specific kinase cascades, so called MAPK modules (22, 23). The MAPK module is composed of three kinases, a MAPK kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK), and a MAPK. These enzymes sequentially phosphorylate and activate each other. The MAPKKKs respond to regulatory signals from various upstream kinases and cell surface receptors. The known MAPKKKs of the JNK family include the MEKK subfamily (MEKK1–4; Refs. 24–26) as well as TAK, DLK, ASK1, MLK, and NCK-interacting kinases (for review, see Ref. 27). The known MAPKKs for the JNK family pathway are JNKK1, also known as SEK1 or MKK4, and JNKK2, also known as MKK7 (28–31). How the various MAPK modules receive and transmit extracellular signals is yet not fully understood.

The proteins that directly interact with JNK can be divided into two groups, those that regulate JNK activity (upstream kinases, inhibitors, and scaffold proteins) and those whose activity is regulated by JNK (substrates). Thus, identification of novel JNK-associating proteins could bring valuable information of the role and function of JNK in various cellular processes. One of the earliest reports of JNK describes it as a kinase that associates with c-Jun and phosphorylates it (18). The association of JNK with c-Jun precedes the phosphorylation reaction and is clearly important for its specificity (32). c-Jun phosphorylation in turn leads to increased transcriptional activity of AP1 (33). JNK associates with most of its other known substrates, like ATF-2, Elk-1, p53, Bcl-2, Bcl-x, and pRb (34–37). Another group of JNK-associating proteins consists of catalytic activators (upstream kinases), non-catalytic activators (scaffold proteins), or inhibitor proteins. JNK-interacting protein, JIP, has been identified as a protein that associates with JNK using the yeast two-hybrid method (34–38). Later, JIP-1 has also been shown to bind JNKK2 and MLK and has been suggested to function as a scaffold for JNK activation (39). Additionally, two other JNK scaffold proteins have been identified, JSAP-1 and β-arrestin 2 (40, 41). β-Arrestin 2 interacts specifically with JNK3 and ASK1. JSAP-1, which appears to be a splicing variant of another JIP family member JIP-3, can bind JNK and is suggested to assemble the MEKK1/JNKK1/JNK3 MAPK module (40). The members of MEKK family can also actually associate directly with JNK and JNKK (42–44), and thus, it has been suggested that the JNK-MAPK module involving MEKKs may function without a scaffold protein (43).

Common for these three reported JNK scaffold proteins is that they enhance JNK activation when overexpressed together with their specific partner molecules. Ectopic expression of β-arrestin 2 has been shown to induce the activity of the cytosolic JNK3 upon co-expression with ASK1, suggesting that the actual function of β-arrestin 2 could be to retain JNK3 in the cytosol and, thus, inhibit its nuclear entry (41). A similar function has been suggested for JIP-1, which was originally cloned as a cytoplasmic inhibitor of JNK due to its ability to associate with JNK and retain it in the cytosol (38). Additionally, proteins such as p21WAF1, pRb, and Hsp72, have been reported to bind JNK directly and inhibit its activity (45–47).

In this study we used the Ras recruitment system (RRS) (48, 49), with the objective to isolate novel JNK-associating proteins. JNK associates relatively tightly with its substrates, and after phosphorylation it rapidly dissociates from them (19, 32, 36). To obtain a more stable association between JNK and its potential substrates, we used a kinase-defective JNK mutant as bait to screen a myristoylated HeLa cell cDNA expression library.

Five cDNA clones encoding proteins that specifically associated with JNK were isolated. The one showing the strongest association with JNK encoded a C-terminal fragment of IKAP (amino acids1040–1333) termed 102-IKAP. We confirmed the association of IKAP and JNK *in vivo*. Furthermore, we showed that the 102-fragment of IKAP could serve as a JNK substrate *in vitro*. Using confocal microscopy with three different detection methods, we found the vast majority of IKAP to localize in the cytosol. Co-expression of IKAP with MEKK1 or ASK1 enhanced JNK activation strongly and specifically. Importantly, ectopic expression of IKAP stimulated neither MEKK1-inducible IKK nor ASK1-inducible p38 activation. Moreover, IKAP efficiently potentiated JNK activation induced by various extracellular stimuli including ultraviolet light irradiation and treatments with either tumor necrosis factor (TNF) or epidermal growth factor.

We mapped the JNK association site in IKAP to its very C terminus. Overexpression of the C-terminal fragment of IKAP, 102-IKAP, was sufficient to induce JNK activity in co-operation with MEKK1. Furthermore, ectopic expression of a C-terminal deletion mutant of IKAP, IKAPΔC, which lacks the JNK association site, was not capable of further facilitating the MEKK1 or ultraviolet light-induced JNK activity. Thus, we showed that the C terminus of IKAP was responsible for the facilitation of JNK activity. Interestingly, this part of IKAP is deleted in sensory, sympathetic, and some parasympathetic neurons of patients affected by familial dysautonomia, an autosomal recessive disease characterized by widespread sensory and variable autonomic dysfunction (5, 6).

EXPERIMENTAL PROCEDURES

cDNA Cloning and Plasmid Constructions—IKAP fragment 102 was cloned using the yeast two-hybrid method, the RRS (48, 49). The myristoylated yeast expression library was constructed from mRNA derived from HeLa cells induced with TNF and interleukin-1 for 30 min. The mRNA was primed with an oligo-dT primer containing an *Xho*I site and reverse-transcribed, and the resulting cDNA was ligated to *Eco*RI linkers. The cDNA was digested with *Eco*RI and *Xho*I and inserted into pYesM Δ poly(A) plasmid (49). The library consisted of 4 million independent transformants. The deletion mutants of 102-IKAP, 102-ΔC-IKAP and 102-RI-IKAP, were prepared by fusing corresponding fragments, encoding amino acids 1040–1216 and 1040–1279, respectively, in-frame with the v-Src myristoylation sequence into pYes expression vector. As bait we used dominant negative JNK1 (JNK1-KM; a gift from F. Dolfi), which was amplified from the HA-tagged pCMV5 expression vector coding for the JNK1-KM by polymerase chain reaction. The PCR product was cloned in-frame with mammalian Ras at its N terminus in alcohol dehydrogenase-Ras yeast expression vector (49) using *Xma*I and *Hin*dIII sites. Corresponding yeast expression constructs were also made from the wild type JNK1. All yeast manipulations and screenings were performed as described (49). 102-IKAP was also subcloned into the eukaryotic expression vector pCDNA3.1His in-frame with the His/Xpress tag at *Bam*HI and *Not*I restriction sites. The 102-IKAP insert was

amplified by PCR using oligonucleotides IK-Myc-2 (5′-CGG GAT CCA TGA ACT TTA CCA AAG AC-3′) and IK-*Not*I (TCC TAA CTG CGG CCG CTC AGT CTA GCA GG-3′) to introduce it into the pCDNA expression vector. Full-length IKAP was obtained with polymerase chain reaction as two fragments utilizing the internal *Xba*I restriction site by using oligonucleotides IK-BamA (5′-CGG GAT CCA TGC GAA ATC TGA AAT TAT TTC GGA C-3′) and IK-XbaA (5′-AGA AAT CTG GTT ACT GGC ATC TAG AAC-3′) for the 5′ fragment and IK-XbaB (5′-TGA CCT TGC TGT TCT AGA TGC CAG TA-3′) and IK-*Not*I (described above) for the 3′ fragment according to the published IKAP sequence (1), so that both PCR products would together contain the full open reading frame of IKAP. A HeLa cell cDNA library (50) was used as template. The obtained fragments were directly subcloned into the pXMyc expression vector between *Bam*HI and *Not*I restriction sites. All 10 individual clones were sequenced, and 1 of the clones, pXMyc6–5, was chosen for further subcloning and analysis.

IKAP was then cloned into pCDNA3.1His eukaryotic expression vector (Invitrogen®, catalog number V385–20). The IKAP cDNA was amplified by PCR using an upstream primer that generated a *Bam*H1 site to fit the reading frame of the His/X-press tag of pCDNA3.1/HisA. The PCR product was digested with *Bam*H1/*Xba*I and *Xba*I/*Not*I, and the two fragments was cloned into pCDNA31./HisA between *Bam*HI and *Not*I sites. This PCR strategy removed the first eight codons of the IKAP reading frame. Primer sequences were as follows: upstream primer (5′-CGGATCCTGGAGTTCAGGGATATTCA-3′) and downstream primer (5′-TCC TAA CTG CGG CCG CTC AGT CTA GCA GG-3′). The IKAP C-terminal deletion construct pCDNA3.1His-IKAPΔC was also made by a PCR-based strategy. The pCDNA3.1His-102-IKAP was made with PCR using oligos Bam-IK2 (5′-CGG GAT CCA TGA ACT TTA CCA AAG-3′) for the upper strand and IK-NotI (described above) for the lower strand amplification. The HA-tagged IKAP C-terminal fragment (used in *in vitro* translation) was made by utilizing the endogenous *Nco*I site in IKAP, and thus, it contains amino acids 1070–1333 of IKAP fused in-frame with the HA tag of pSRα3, analogous to pSRα3-HA-c-Jun also used in this study. All the clones were verified by sequencing and Western blotting. To generate an in-frame C-terminal fusion of IKAP to green fluorescent protein (GFP), we amplified His/X-press-tagged IKAP cDNA from pCDNA3.1HisIKAP using primers 5′-GTCGACCCAAGCTGGCTAGCGTTTAAAC-3′ and 5′-TAGATCTAGC-AGGCTCAGCTTCCACTGG-3′ and inserted it as a *Sal*I/*Bgl*II restriction fragment between the *Xho*I and *Bam*H1 sites of pEGFP-N1 (CLONTECH laboratories, Inc., catalog number PT3027-5). We term this plasmid pCMV-IKAP/GFP. All plasmid construction was verified by sequencing.

Expression vectors for HA-JNKI wild type and dominant negative, APF-JNK1, the corresponding JNK2 constructs, and HA-c-Jun have been described elsewhere (17, 19). Dominant negative KM-JNK1 was a gift from F. Dolfi. Expression constructs for GST-c-Jun-(1–79) (18), MEKK1Δ (43), and dominant negative HA-tagged IKKα and -β (51) were obtained from M. Karin.

Cell Culture and Transfections—HeLa and 293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Invitrogen). Plasmid DNA was transfected into 293 cells using the DNA/calcium phosphate co-precipitation method and into HeLa cells with FuGENE 6 (Roche Molecular Biochemicals) lipid carrier according to the manufacturer's protocol. After 36 h the cells were either harvested, lysed, pelleted and the supernatants were recovered for further analysis, or they were fixed and immunostained as described in experimental procedures below.

Antibodies and Reagents—TNF-α was a gift from Dr. A. Cerami, nickel-agarose was purchased from Qiagen, and protein A-, G-, and GSH-Sepharose were purchased from Amersham Biosciences. Anti-HA antibody was purified from hybridoma 12CA5. Anti-His antibody and anti-X-press antibodies were from Invitrogen. JNK antibodies 333.8 and 666.3 were obtained from BD PharMingen, and the anti-MEKK1 (C22) and anti-IKAP (H302) were from Santa Cruz. Isotopes were from Amersham Biosciences.

Hybridization Analysis—The human multiple tissue expression array membrane (CLONTECH) was hybridized to [α-^{32}P]dATP-labeled IKAP cDNA probe according to the manufacturer's protocol. The probe was prepared by labeling IKAP cDNA with [α-^{32}P]dATP (Amersham Biosciences) with the random prime labeling method.

In Vitro Translation—*In vitro* translation of HA-c-Jun, HA-IKAP C-terminal fragment, and HA-E2F1 were performed according to the manufacturer's protocol using the TNT-coupled reticulocyte lysate and SP6 RNA polymerase (Jun and IKAP) or T7 polymerase (E2F1). The efficiency of the translation reaction was first estimated by incorporation of ^{35}S-labeled methionine. The actual *in vitro* translation reaction to produce HA-tagged c-Jun, IKAP, and E2F1 substrates was carried out with 25 mM cold methionine. The HA-immunoprecipitated proteins were tested as JNK substrates in standard *in vitro* kinase assays.

Immunofluorescence—HeLa cells were fixed onto cover slips with ice-cold methanol/ethanol for 10 min before drying and subsequent rehydration in PBS. Unspecific epitopes were blocked with 10% fetal calf serum, 1% bovine serum albumin, 0.3% Triton X-100 in phosphate-buffered saline for 1 h at room temperature. To detect JNK we used antibody 666 from BD PharMingen diluted 1:3000. Anti-IKAP (H302 from Santa Cruz Biotechnology) was diluted 1:200. IgG controls were used in the same concentrations. Primary antibodies were allowed to bind for 1 h at room temperature, after which the cover slips were washed 3 times for 10 min in phosphate-buffered saline, 0.25% bovine serum albumin, 0.1%Triton X-100. Secondary antibodies from Molecular Probes (Alexa Fluor® 594 donkey anti-mouse for JNK and Express-tagged IKAP and Alexa Fluor® 488 donkey anti-rabbit for IKAP) were diluted 1:2000 and allowed to bind for 30 min. After three washes the cover slips were mounted onto glass slides using Prolong® Antifade (Molecular Probes) as medium. HeLa cells expressing the IKAP/green fluorescent protein fusion were fixed in 4% paraformaldehyde before mounting. Fluorescence microphotographs were generated on a Zeiss confocal microscope.

Co-precipitations and Kinase Assays—For the co-precipitation of endogenous IKAP and JNK, the cells were lysed in 50 mM Tris, pH 7.6, 100 mM NaCl, 3 mM EDTA, and 0.1% Triton X-100 in the presence of 1 mM dithiothreitol, and 10 μg/ml aprotinin, leupeptin, and pepstatin A by passing through a 26-gauge needle 6 times. Soluble extracts were prepared by centrifugation in a microcentrifuge at 15,000 rpm for 15 min at 4 °C. The lysates were collected and diluted with 3 volumes of 50 mM Tris, pH 7.6, and 0.5 mM dithiothreitol. The lysates were incubated with anti-JNK antibody (333.8) and the protein A/G-Sepharose mixture at 4 °C with slow rotation for 2 h, and the Sepharose beads were washed with 50 mM Tris, pH 7.6, 50 mM NaCl, 1 mM EDTA, and 0.15% Nonidet P-40. The precipitates and associated proteins were resolved on 8% SDS-PAGE, transferred to Hybond ECL-nitrocellulose (Amersham Biosciences), and hybridized with anti-IKAP antibody (H302), and the hybridized proteins were detected using ECL-Western-blotting reagents (Amersham Biosciences).

The cell extracts used in the immunocomplex kinase assays were prepared as described. The cells were lysed by incubating them for 20 min at 4 °C in radioimmune precipitation assay lysis buffer supplemented with 1 mM *p*-nitrophenyl phosphate, 10 mM β-glycerophosphate, 100 μM sodium -vanadate, and 1 μg/ml each aprotinin, leupeptin, and pepstatin A. Soluble extracts were prepared by centrifugation in a microcentrifuge at 15,000 rpm for 15 min at 4 °C. For precipitations and association assays, the extracts were diluted with 2.5 volumes of whole cell extract buffer (18) and incubated with designated antibodies and the protein A/G-Sepharose mixture at 4 °C with slow rotation for 2 h. Precipitates were washed four times with Hepes balanced binding buffer (18) and once with JNK buffer (19). The phosphorylation reactions were allowed to proceed in JNK buffer for 20 min at 30 °C using 10 μg of designated substrate, 1 μM ATP, and 5 μCi of [γ-^{32}P]ATP in 30 μl of total volume. The reactions were terminated with Laemmli sample buffer and boiled, and the products were resolved on 12% SDS-PAGE. JNK activity was quantified as phosphorylated substrate by phosphorimaging (Molecular Dynamics).

RESULTS

Cloning of IKAP; JNK Associates with IKAP in Vivo and Phosphorylates It in Vitro—To identify novel cytoplasmic JNK-associating proteins, we have used the alternative yeast two-hybrid method, the RRS (48, 49). In RRS, the association between bait and prey occurs in the cytosol. Because of the transient nature of the kinase-substrate association, we used a catalytically inactive form of JNK as bait. A similar approach has been used previously to identify MEF2C as a p38 substrate employing the conventional two-hybrid system (52). The JNK bait was designed to encode for a catalytically inactive JNK1 (JNK1-KM) fused to Ras at its N terminus (JNK1-Dn.Ras(61)ΔF). We screened about 10% of the myristoylated expression library (53) made from mRNA isolated from TNF-α and interleukin 1-stimulated HeLa cells.

Five individual colonies exhibited efficient growth at the restrictive temperature when grown on galactose-containing medium. DNA extracted from these clones was reintroduced

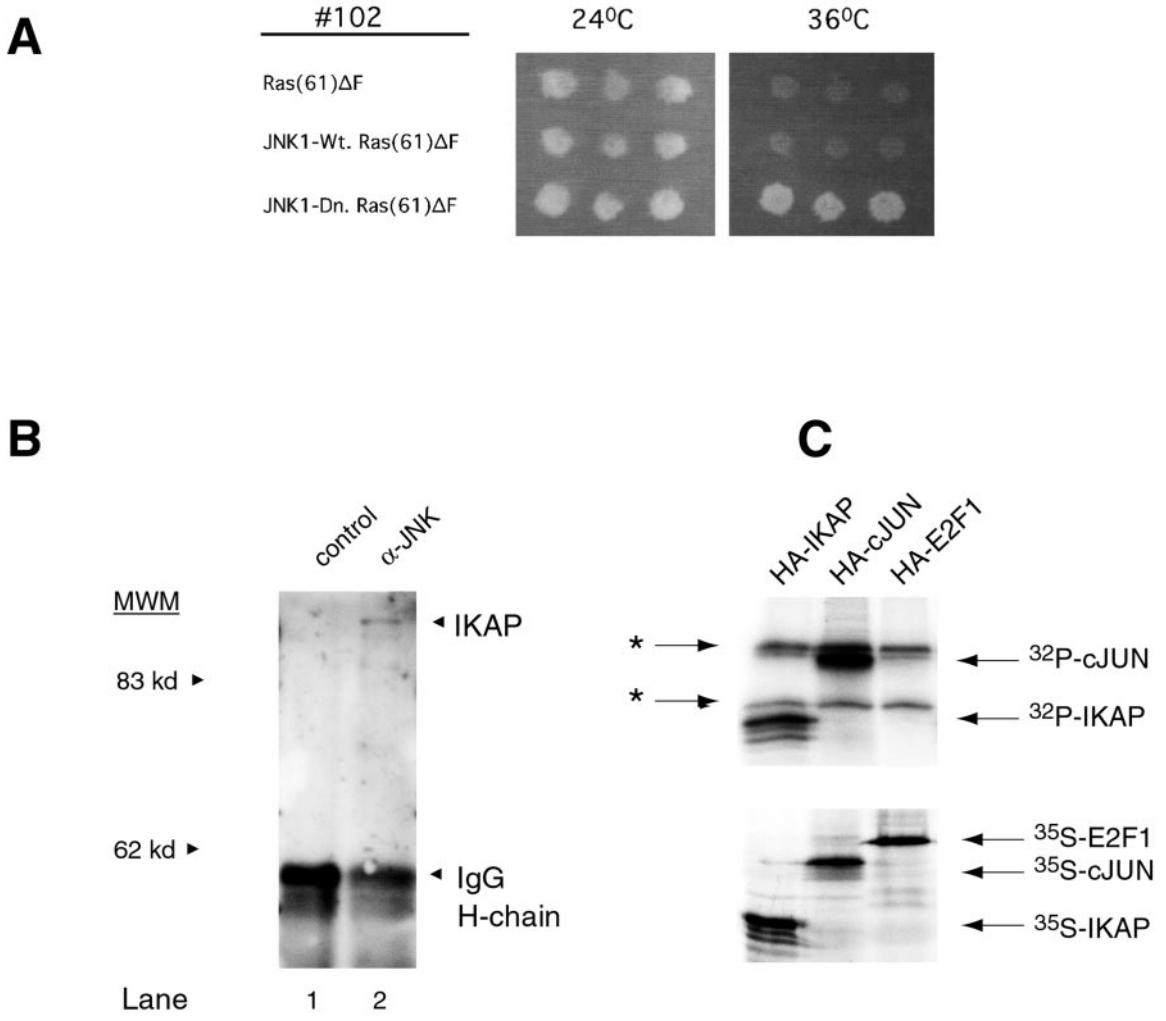

FIG. 1. **JNK associates with IKAP and phosphorylates it.** *A*, JNK associates with IKAP in the yeast Ras recruitment system. Yeast *S. cerevisiae* Cdc25-2 cells were co-transfected with plasmids encoding for IKAP-102 fused to v-Src myristoylation signal together with cytoplasmic Ras (*Ras (61)ΔF*), JNK1 wild type bait (*JNK1-Wt.Ras(61)ΔF*) or inactive JNK1 bait (*JNK1-Dn.Ras(61)ΔF*). Transformants were selected and grown at 24 °C for 2 days, replica-plated onto galactose-containing medium, and incubated at 36 °C. *B*, endogenous IKAP can be co-precipitated with anti-JNK antibody. One mg of cytosolic extract from MCF-7-S1 cells was precipitated with either a nonspecific control antibody (α-Myc monoclonal antibody 9E10) (*lane 1*) or the JNK antibody 333.8 (*lane 2*). The precipitates were run on 8% SDS-PAGE, and the proteins were blotted on nitrocellulose and hybridized with anti-IKAP antibody. The JNK-specific antibody co-precipitated a 150-kDa protein, which was recognized with the anti-IKAP-antibody. *C*, JNK can phosphorylate IKAP *in vitro*. HA-tagged c-Jun, 102-IKAP, and E2F1 were *in vitro* translated and precipitated with anti-HA antibody, and the precipitates were tested as substrates of recombinant active JNK in the presence of [γ-^{32}P]ATP. The phosphorylated proteins were resolved on 12% SDS-PAGE and autoradiographed. JNK phosphorylated HA-102-IKAP (*lane 1*) as efficiently as HA-c-Jun (*lane 2*), whereas no phosphorylation of HA-E2F1 could be detected (*lane 3*). Two phosphorylated nonspecific bands from the bacterial lysate could be seen in all the samples (marked with *asterisks*). The *lower panel* shows the amount of input protein.

into yeast cells in the presence or absence of the original bait. All five plasmid DNAs resulted in efficient JNK1-Dn-dependent growth (data not shown). We focused on the clone 102, which exhibited the strongest binding to JNK1 and showed most potent growth at the restrictive temperature. Sequence analysis revealed that it encoded the C-terminal part of IKAP starting at the amino acid 1040 of the published sequence (1).

In yeast, 102-IKAP specifically associated with kinase inactive JNK (KM-JNK) in which the critical lysine residue in the ATP binding site was replaced with methionine. No association with wild type JNK1, JNK1-Wt.Ras(61)ΔF, or Ras (61)ΔF alone was observed (Fig. 1*A*). To detect a possible association between the endogenous IKAP and JNK, we prepared lysates from semi-confluent MCF7-S1 cells and used the JNK-specific antibody (333.8) to precipitate the endogenous JNK. After several washes, the precipitates were run on SDS-PAGE and subjected to Western blotting. The blot was hybridized with an IKAP-specific antibody (H-302); a 150-kDa protein was observed to co-precipitate in anti-JNK-precipitated cell extract but not in the control precipitation (Fig. 1*B*; compare *lanes 1* and *2*).

To test whether IKAP can serve as a JNK substrate *in vitro*, a recombinant active JNK (32) was used to phosphorylate the *in vitro* translated HA-tagged C-terminal fragment of IKAP. Despite our attempts, we were unable to express 102-IKAP in bacteria. Thus, *in vitro* translated immuno-purified HA-tagged C-terminal IKAP fragment, HA-c-Jun, and HA-E2F1 were used in a kinase assay with bacterially purified active His-tagged JNK2 in the presence of [γ-^{32}P]ATP. The phosphorylated proteins were resolved by 12% SDS-PAGE, dried, and autoradiographed. JNK efficiently phosphorylated both IKAP and c-Jun (Fig. 1*C*, *upper panel*; *first* and *second lanes*) but not E2F1 (*third lane*), indicating that IKAP can serve as a JNK substrate *in vitro*. The *lower panel* of Fig. 1*C* shows the amount of input protein in the kinase assays. The extent of HA-IKAP phosphorylation was similar to that observed with HA-c-Jun. Indeed, this IKAP fragment contains two potential JNK consensus proline-directed phospho-acceptor sites, SP (amino acid 1211) and TP (amino acid 1284). We cloned the cDNA encoding the

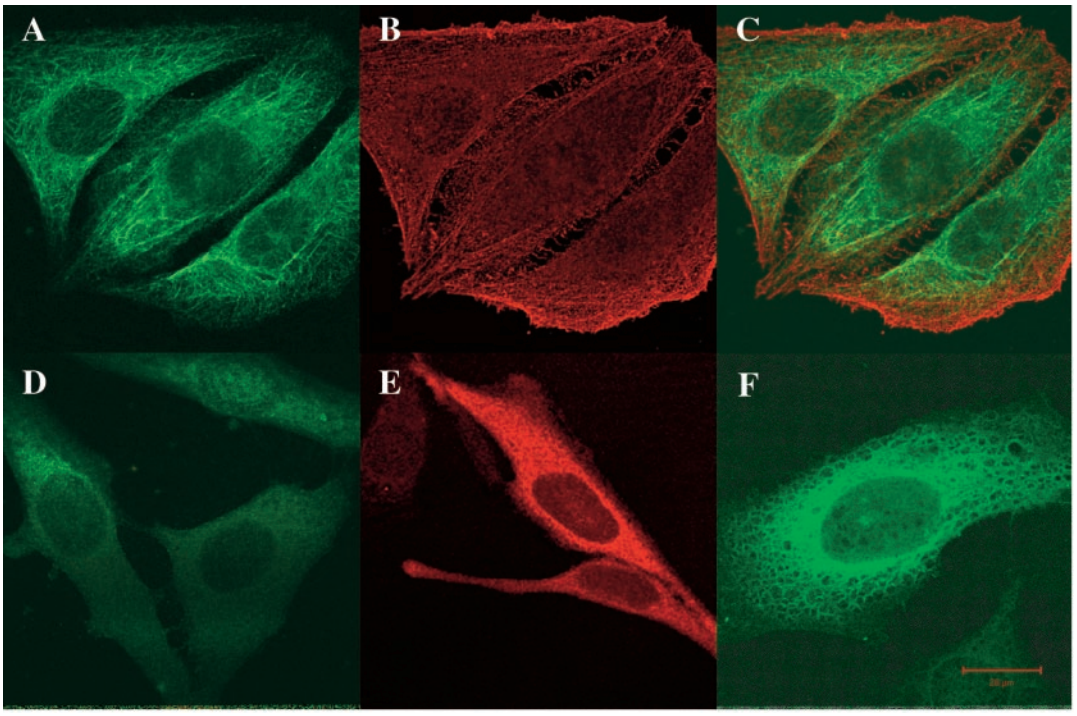

FIG. 2. **IKAP localizes to the cytosol.** Immunofluorescent stains for JNK and IKAP in HeLa cells. *A*, anti-IKAP staining (H302 polyclonal from Santa Cruz Biotechnology) showing the cytosolic localization of the endogenous IKAP. *B*, anti-JNK staining (666 monoclonal from BD PharMingen, recognizing both JNK1 and JNK2), demonstrating JNK localization in membrane, cytosol, and nucleus. *C*, overlay of JNK and IKAP staining. *D* shows IgG negative controls for mouse and rabbit as *red* and *green* overlays. *E* shows the localization of ectopic IKAP carrying an X-press tag, using the monoclonal anti-X-press antibody from Invitrogen. Untransfected neighboring cells serve as negative controls. *F* shows cytosolic localization of the IKAP/green fluorescent protein fusion protein. The *scale bar* is 20 μm.

full-length IKAP protein by a reverse transcriptase polymerase chain reaction using a HeLa cell cDNA library as template. We sequenced 10 individually obtained clones. All of them differed from the published sequence (1) by four nucleotides (bp, 423 T > C; bp 1144, C > T; bp 1168, A > G; and bp 2494, C > T). This probably reflects the variability between HeLa cells from different laboratories. Three of these changes also provoked alterations in the amino acid sequence, but they did not lie close to any of the earlier mapped IKAP association sites.

IKAP Is a Cytosolic Protein That Partially Co-localizes with JNK—To test whether endogenous JNK and IKAP co-localize in the cell, we co-stained HeLa cells with specific antibodies against JNK (333.8) and IKAP (H-302), respectively, and analyzed for co-localization using fluorescence confocal microscopy. As evident from Fig. 2*A*, IKAP stains in a thread-like pattern in the cytoplasm, with most intense staining in the perinuclear area. The JNK-specific antibody stained strong in the membrane, cytoplasm, and to a minor degree, in the nucleus (Fig. 2*B*). Overlay of the two stains revealed partial co-localization (Fig. 2*C*) in the cytosol around the nucleus. Little co-localization was observed elsewhere. When His/X-press-tagged IKAP was overexpressed in HeLa cells and the cells were immunostained against the X-press epitope tag, the IKAP localization appeared again exclusively cytoplasmic (Fig. 2*E*). A similar pattern was also observed using ectopically expressed green fluorescent protein-tagged IKAP (Fig. 2*F*). These observations are supported by an earlier study in which IKAP was also shown to localize mainly in the cytosol using cell fractionation and Western blot analysis, utilizing a different IKAP antibody from what we use in this study (2). We conclude that the vast majority of IKAP is cytoplasmic.

IKAP Is Widely Expressed in Various Human Tissues and Cell Lines—To examine the tissue distribution of IKAP we hybridized the multiple tissue expression array blot (CLONTECH) to a ^{32}P-labeled IKAP cDNA probe (Fig. 3, *A* and *B*). We quantified the hybridization signal obtained from the different tissues by phosphorimaging. The weakest hybridization was observed in ovaries and set to one; all other hybridization values are presented relative to that. In adult tissues, the strongest expression of IKAP mRNA was observed in the cerebellum. The hybridization value shown (19.6) for cerebellum is the mean of the values for the left (20.8) and right (18.4) lobes. Relatively high expression of IKAP mRNA was also observed in the pituitary gland, adrenal gland, liver, and kidneys. Relative expression of IKAP in whole brain fraction is presented in both figures. Apart from the high expression in cerebellum and pituitary gland, IKAP expression in the remaining brain compartments was comparable with that in most other tissues (Fig. 3, *A* and *B*).

IKAP Can Specifically Facilitate JNK Activation Induced by Various Divergent Stimuli—Because IKAP associates with JNK, we examined whether it can have a regulatory role and whether the ectopic expression of IKAP can affect JNK activity. Therefore, we co-expressed HA-tagged JNK1 in 293 cells with or without His/X-press-tagged IKAP (for the expression pattern, see Fig. 2*E*) in the absence or presence of increasing concentrations of MEKK1Δ, a most potent activator of JNK (54). JNK activation was measured by immunocomplex kinase assay using GST-c-Jun (1–79) as a substrate. The overexpression of IKAP alone moderately activated JNK (Fig. 4*A*; compare *lanes 1* and *5*). Overexpression of IKAP together with low concentrations of MEKK1Δ resulted in further potentiation of JNK1 activity (Fig. 4*A*; *upper panel*, compare *lanes 2* and *3* with *lanes 6* and *7*). Importantly, the expression levels of HA-JNK1 were not affected by IKAP (Fig. 4*A*; *lower panel*). The graphic quantification presents the mean of three independent experiments. To determine the significance of this finding, we calculated the mean and S.D. of 5 JNK assays from 5 independently transfected 293 cell populations. The effect of IKAP in the potentiation of MEKK1Δ-induced JNK activation was found highly significant ($p = 0.0018$) in Student's t test (Fig. 4*B*).

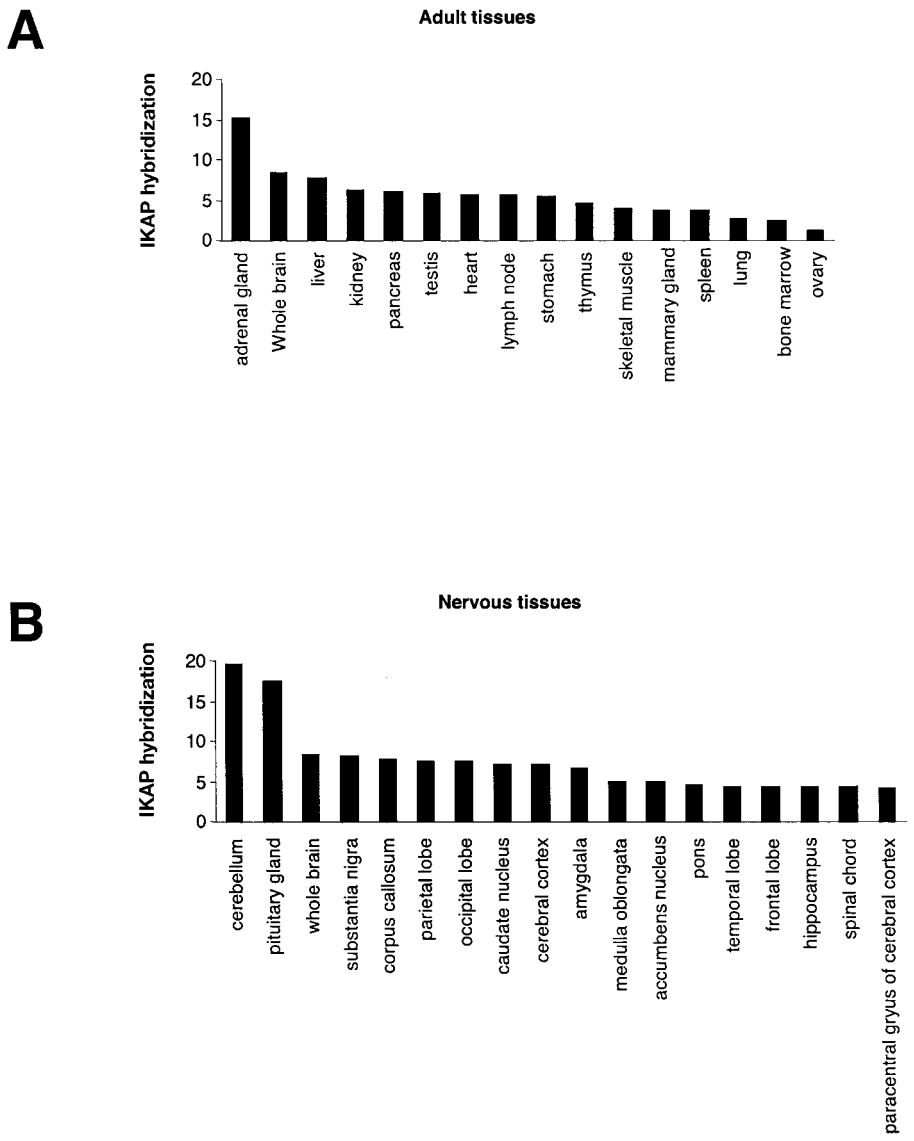

FIG. 3. **IKAP is ubiquitously expressed.** *A*, IKAP expression in various adult tissues. Multiple tissue expression array membrane (CLONTECH) was hybridized to ^{32}P-labeled IKAP cDNA fragment. IKAP expression was quantified using phosphorimaging. The lowest expressing tissue, ovary, was chosen to have a relative hybridization value of 1, and the IKAP expression level of the rest of the tissues were normalized accordingly. *B*, IKAP expression in nervous tissues. The IKAP expression in nervous tissues was quantified and normalized as in *A*.

As a specificity control we tested the ability of IKAP to facilitate MEKK1-induced IKK activity. Co-expression of the His/X-press-tagged IKAP with HA-IKKβ together with increasing concentrations of MEKK1Δ (Fig. 4*C*; *top panel*, *lanes 1–4*) did not result in any measurable effect on the activity of IKKβ as compared with the vector control (Fig. 4*C*; *top panel*, *lanes 5–8*). Again, ectopic expression of HA-IKKβ was not affected by IKAP (Fig. 4*C*; *lower panel*).

We also tested the ability of IKAP to facilitate ASK1-induced JNK activation in a similar experimental setting (Fig. 4*D*). Co-expression of the His/X-press IKAP with the increasing concentrations of ASK1 (Fig. 4*D*; *top panel*, *lanes 1–4*) clearly potentiated JNK activity compared with the vector control (Fig. 4*D*; *top panel*, *lanes 5–8*). The quantifications represent the means of three independent experiments. The expression of FLAG-JNK1 was unaffected by IKAP (Fig. 4*D*, *lower panel*). To determine the specificity of IKAP induction on JNK pathway, we also co-expressed the His/X-press IKAP together with the HA-tagged p38 MAP kinase together with increasing concentrations of ASK1. Activation of p38 was measured as phosphorylation of GST-ATF2 (Fig. 4*E*, *upper panel*). IKAP could not further facilitate ASK1-induced p38 activity. The expression level of the HA-p38 was unaltered upon IKAP expression (Fig. 4*E*, *lower panel*).

Finally, we also tested the ability of IKAP to facilitate JNK activity induced by extracellular stimulus. For this we transfected HeLa cells with HA-JNK1 in the presence (Fig. 4*F*, *lanes 1–4*) or absence of IKAP (Fig. 4*F*, *lanes 5–8*). Cells were treated with the indicated doses of the known JNK activators, TNF, epidermal growth factor, or 100 J/m^2 UV-C light for 20 min before harvesting. Immunocomplex kinase assays were performed using GST-c-Jun-(1–79) as a substrate. In these experiments IKAP strongly promoted JNK activity induced by all these divergent extracellular stimuli (Fig. 4*F*; compare *lanes 1–4* with *lanes 5–8*). We conclude that excess IKAP specifically promotes JNK activation.

Mapping JNK Association Site in IKAP—The IKAP isolated in our screen corresponds to the C-terminal domain of IKAP (amino acids 1040–1333). IKAP was previously shown to interact *in vitro* with NIK, IKKα, and IKKβ at distinct domains between amino acids 441 and 920. We used the RRS to better define the JNK binding domain within IKAP. We designed two C-terminal deletion mutants of the original clone 102, designated 102-ΔC-IKAP (amino acids 1040–1216) and 102-RI-IKAP (amino acids 1040–1279), into pYes expression vector (Fig. 5*A*; *bottom panel*). The different IKAP deletion mutants were co-transfected into Cdc25–2 yeast cells together with the original JNK1-Dn.Ras(61)ΔF bait. Transformants were selected and tested for their ability to grow at the restrictive temperature (Fig. 5*A*, *upper panels*). Transformants expressing

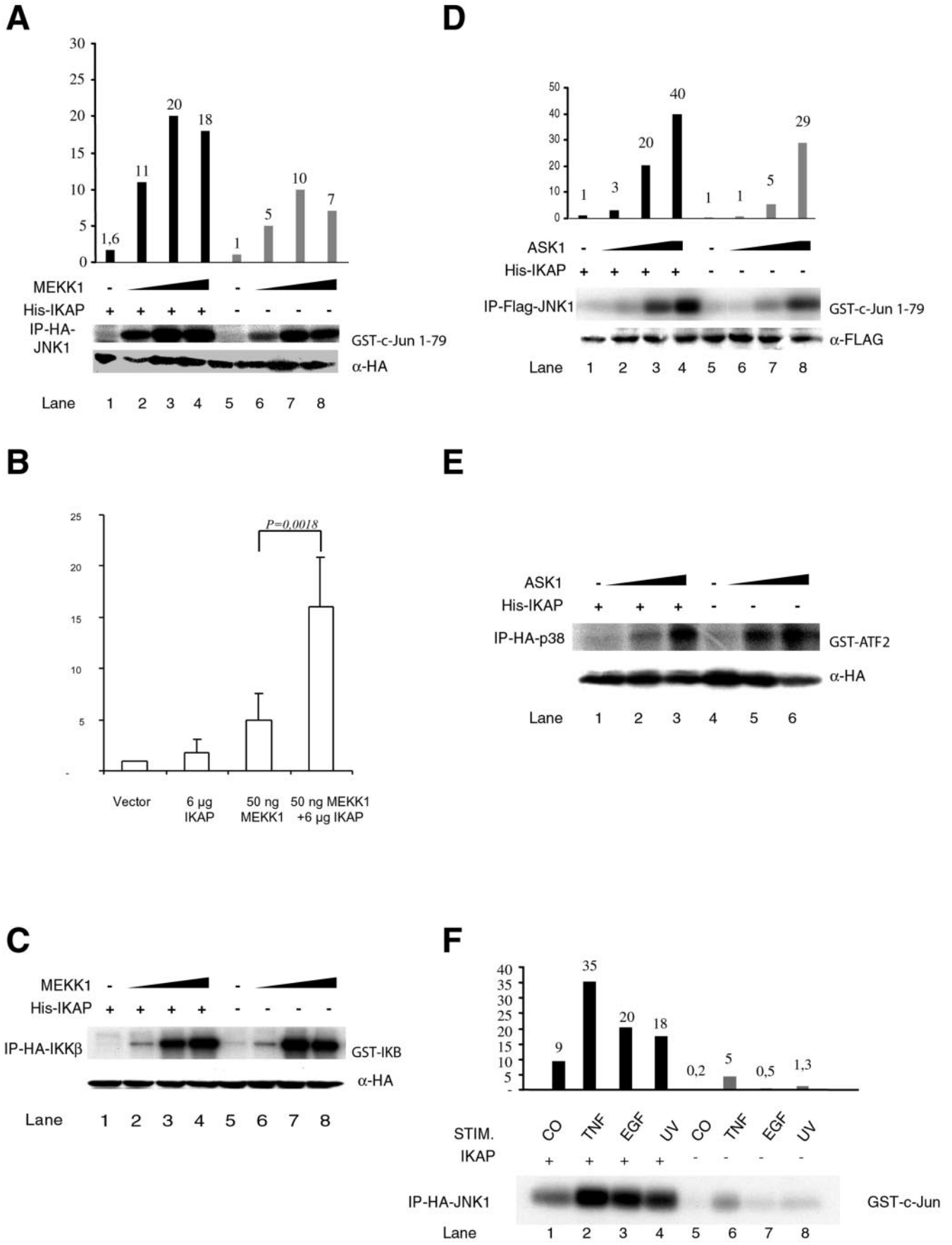

FIG. 4. **IKAP potentiates JNK activity but has no effect on IKKβ or p38 activity.** *A*, IKAP potentiates MEKK1-induced JNK activity. 293 cells were co-transfected with 6 μg of HA-JNK1 expression vector with either 6 μg of His-IKAP (*lanes 1–4*; *black bars*) or 6 μg of empty expression vector (*lanes 5–8*; *gray bars*) together with increasing concentration of MEKK1 (0, 50, 100, and 250 ng) using the calcium phosphate co-precipitation method. JNK was precipitated (*IP*) with HA antibody, and the immunocomplex kinase assays were performed using GST-c-Jun-

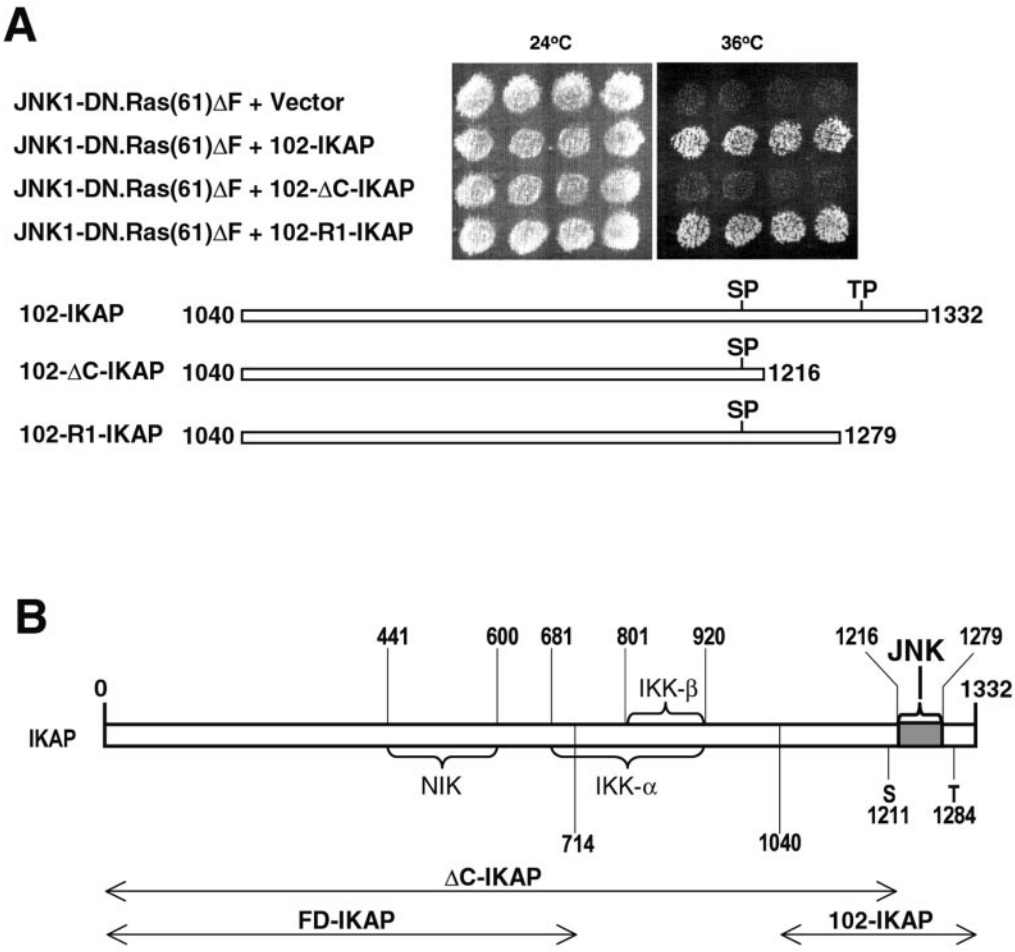

FIG. 5. **Mapping the exact JNK association site in IKAP.** *A*, mapping the JNK association site using RRS. Cdc25–2 cell were co-transfected with ADNS-JNK1-Dn.Ras(61)ΔF bait together with Yes2 empty vector (*Vector*), and IKAP protein fragments corresponding to the indicated amino acids within IKAP are described in the scheme at the *lower panel*. Transformants were selected and grown at 24 °C for 2 days and subsequently replica-plated onto galactose-containing plates and incubated at 36 °C. *SP*, and *TP*, potential proline-directed serine/threonine JNK phosphorylation sites, respectively. *B*, schematic representation of IKAP protein. The JNK association site (*gray box*) is described and mapped in this study. The suggested IKK and NIK association sites within IKAP, according to previously published data (1), are shown. The position of the original IKAP clone 102 isolated with the two-hybrid screen as well as the FD-IKAP protein, the short splicing form of IKAP expressed in familial dysautonomia, are indicated. The two potential proline-directed serine/threonine JNK phosphorylation sites around the JNK association site are also shown.

the IKAP fragment encoding for amino acids 1040–1216 were unable to grow, whereas transformants expressing IKAP fragment 1040–1279 exhibited efficient growth at the restrictive temperature, similar to the original clone. Thus, the domain important for JNK association within IKAP resides between amino acids 1216 and 1279 and is clearly distinct from the previously suggested IKKα, IKKβ, and NIK binding sites, and most importantly, it is not included in familial dysautonomia (FD)-IKAP, the IKAP deletion mutant involved in the familial dysautonomia (Fig. 5, *A* and *B*).

The C Terminus of IKAP Is Sufficient for the Promotion of JNK Activation—The fact that 102-ΔC-IKAP no longer associates with JNK in yeast prompted us to test whether truncation of the JNK association domain within IKAP will affect the JNK

(1–79) as a substrate (*top panel*). Kinase assays were quantified on a PhosphorImager. Expression levels of the HA-JNK1 are shown in the anti-HA Western blot (*lower panel*). *B*, IKAP significantly potentiates MEKK1-induced JNK activation. 293 cells were co-transfected with 6 μg of HA-JNK expression vector together with His-IKAP and MEKK1 expression vectors as indicated. Total DNA amount was kept constant by the addition of empty expression vector. JNK activity was assayed as in *A* and normalized to the vector control. The means ± S.D. of five independent experiments are shown. The statistical significance was analyzed using a two-tailed Student's *t* test. *C*, IKAP has no effect on the MEKK1-induced IKKβ activity. 293 cells were co-transfected with 6 μg of HA-IKKβ expression vector with either 6 μg of His-IKAP expression vector (*lanes 1–4*) or 6 μg of empty expression vector (*lanes 5–8*) together with increasing concentrations of MEKK1 (0, 500 ng, 1 μg, 2 μg). HA-IKKβ was precipitated with HA antibody, and the immunocomplex kinase assays were performed using GST-IKB as a substrate. Expression of the HA-IKKβ was determined by Western blotting (*lower panel*). *D*, IKAP potentiates ASK1 induced JNK activity. 293 cells were co-transfected with 6 μg of FLAG-tagged JNK1 with either 6 μg of His-IKAP (*lanes 1–4*) or 6 μg of empty expression vector (*lanes 5–8*) together with increasing concentrations of ASK1 (0, 50, 100, 250 ng). JNK was immunoprecipitated with FLAG-antibody, and the immunocomplex kinase assays were performed using GST-c-Jun-(1–79) as a substrate (*top panel*). Kinase assays were quantified with phosphorimaging, and the expression of FLAG-JNK1 was determined using FLAG antibody (*bottom panel*). *E*, IKAP does not facilitate the ASK1-induced p38 activity. HeLa cells were co-transfected with 1.5 μg of p38 with either 1.5 μg of His-IKAP (*lanes 1–3*) or 1.5 μg of empty expression vector (*lanes 4–6*) using FuGENE 6 transfection reagent together with increasing concentrations of ASK1 (0, 1, 2 μg). HA-p38 was precipitated using anti-HA antibody, and the immunocomplex kinase assays were performed using GST-ATF2 as a substrate (*top panel*). The expression level of the HA-p38 in each individual case is determined by Western blotting (*lower panel*). *F*, IKAP potentiates JNK activity induced by divergent extracellular stimuli (*Stim.*). HeLa cells were co-transfected with 1.5 μg of HA-JNK1 together with either 1.5 μg of His-IKAP (*lanes 1–4*) or empty expression vector (*lanes 5–8*). JNK activity was either mock-induced or induced by treatments with 100 ng/ml TNF, 50 ng/ml epidermal growth factor, or 40 J/m^2 of UV light. Incorporation of the [γ-^{32}P]ATP was quantified using phosphorimaging.

activation in mammalian cells. We prepared His/X-press-tagged eukaryotic expression vectors encoding for the IKAP deletion mutants 102-IKAP, a C-terminal fragment of IKAP that contains the JNK binding site, and IKAPΔC, an N-terminal IKAP construct truncated at amino acid 1216 (see Fig. 5*B*). 293 cells were co-transfected together with HA-JNKI and either His/X-press-tagged wild type IKAP, His-102-IKAP, or His-IKAPΔC. First we tested whether the C-terminal fragment of IKAP, 102-IKAP, would suffice for the MEKK1-induced promotion of the JNK activation. We transfected the 293 cells with HA-JNK1 together with His/X-press-102-IKAP or empty expression vector together with increasing concentrations of MEKK1Δ (Fig. 6*A*). 102-IKAP clearly promoted the MEKK1-inducible JNK activity and nearly to the same extent as wild type IKAP, suggesting that the region in IKAP sufficient for the regulation of the JNK activity lies within the 294-most C-terminal amino acids of IKAP (Fig. 6*A*). In the next experiment, the His/X-press-IKAPΔC and wt His/X-press-IKAP were used instead of His/X-press 102-IKAP. In two of the transfections, JNK activity was induced with co-expression of MEKK1Δ (Fig. 6*B*; *lanes 2* and *4*), and in the other two, activity was induced with UV-C irradiation (Fig. 6*B*; *lanes 6* and *8*). In both cases IKAPΔC was not able to promote the UV light or MEKK1Δ-inducible JNK activity to the same level as wild type IKAP (Fig. 6*B*; compare *lanes 2–4* and *lanes 6–8*).

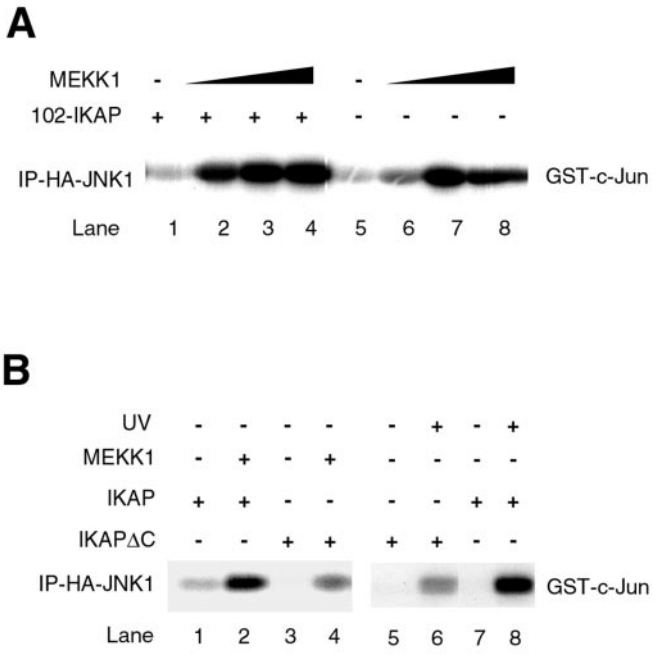

FIG. 6. **C terminus of IKAP is responsible for its capability to facilitate JNK activation.** *A*, the C-terminal fragment of IKAP is sufficient to promote the MEKK1-inducible JNK activity. 293 cells were co-transfected with HA-JNK1 and with either eukaryotic expression vector for the His-102-IKAP (*lanes 1–4*) or empty expression vector (*lanes 5–8*) together with increasing concentrations of MEKK1Δ (0, 50, 100, 250 ng). The HA-JNK1 was immunoprecipitated (*IP*) using anti-HA-antibody, and the immunocomplex kinase assays were performed using GST-c-Jun-(1–79) as a substrate (*top panel*). The expression levels of the HA-JNKs are determined by Western blotting (*lower panel*). *B*, IKAPΔC cannot facilitate either MEKK1 or UV light-induced JNK activity. 293 cells were transiently transfected with 6 μg of HA-tagged JNK1, 6 μg of IKAP (*lanes 1* and *2*), or 6 μg of IKAPΔC (IKAP mutant devoid of JNK association site) in the presence (*lanes 2* and *4*) or absence (*lanes 1*, *3*, *5*, *6*, *7*, *8*) of 50 ng of MEKK1Δ. The cells used for the experiments in *lanes 6* and *8* were exposed to UV light (100 J/m^2) 20 min before harvesting to activate the JNK pathway. The immunocomplex kinase assays were performed using the anti-HA antibody and GST-c-Jun-(1–79) as a substrate.

DISCUSSION

IKAP-JNK Association—IKAP was originally isolated as a 150-kDa protein component of the large interleukin-1-inducible IKK complex containing NIK, IKKα, IKKβ, and NF-κB/relA (1). It was, however, subsequently described as a "not readily detectable component" of the IKK complex as reviewed by Michael Karin in (55). Later, IKAP was shown to associate with unknown 100-, 70-, 45-, and 39-kDa proteins but, notably, not with IKK components (2). In a more recent study, it was shown to associate specifically with 95- and 60 (designated as hELP3)-kDa proteins to form the mammalian RNA polymerase II core elongator (3). Functional elongator has histone acetylase activity, yet neither IKAP nor the other core elongator components possess any histone acetylase activity, and formation of the functional elongator complex requires the association of the three small holo-elongator subunits, hELP4 and the unknown proteins p38 and p30, with the core elongator to support histone acetylase activity of hELP3 (4). Accordingly, IKAP is proposed to have a scaffold-type of function in the elongation process.

We have isolated a C-terminal fragment of IKAP (amino acids 1040–1333) that interacted with inactive JNK1 in the yeast RRS method. Furthermore, we demonstrated that recombinant JNK could phosphorylate *in vitro* translated 102-IKAP, and we confirmed the association *in vivo* as endogenous JNK was able to co-precipitate IKAP. However, we were unable to detect the association of the *in vitro* translated IKAP with recombinant JNK (data not shown), possibly indicating that their stable association in mammalian cells is supported by one or more accessory factors. We mapped the domain within IKAP responsible for JNK binding to amino acids 1216–1279. This domain exhibits no sequence homology to the JNK docking domain found in c-Jun nor does it share sequence homology with docking domains of other JNK substrates. It is also clearly distinct from the earlier reported IKKα, IKKβ, and NIK association sites (Ref. 1; see also Fig. 5*B*).

We show in this study that IKAP can regulate JNK stress kinase activation efficiently in mammalian cells. Ectopic expression of MEKK1 is by far one of the most efficient ways to activate JNK, yet we demonstrated here that expression of low levels of IKAP together with MEKK1 in 293 cells further potentiated the MEKK1-induced JNK activation (2-fold). As controls for specificity, we have shown that IKAP was unable to further facilitate MEKK1-induced IKK activity.

We observed a stronger effect of IKAP on the MEKK1-related ASK1. Here, ASK1-induced JNK activation was elevated 3–4-fold in the presence of excess IKAP. Importantly, IKAP had no positive effect on the ASK1-induced p38 activity, again demonstrating the specificity of IKAP on JNK signaling. The strongest effect of IKAP on JNK activation was detected (up to 7-fold) when various extracellular stimuli were used to induce JNK activity in HeLa cells. Our data clearly show a specific and novel role for IKAP in JNK activation pathways in mammalian cells.

Role of IKAP in Cell Signaling—To have a role in transcriptional elongation, IKAP needs to reside in the nucleus. Yet the sequence analysis algorithm PSORTII (psort.nibb.ac.jp) fails to identify nuclear localization and nuclear export signals in IKAP, and the program predicts IKAP to localize in the cytoplasm with high probability (61%). Indeed, we and others find experimentally that the vast majority of IKAP localizes in the cytoplasm. Nevertheless, weak nuclear staining can also be detected, and it is possible that IKAP shuttles between nucleus and cytosol, as was also suggested by Hawkes and co-workers (4). The fact that IKAP mainly localizes to the cytosol in the resting mammalian cells (see Ref. 2 and Fig. 2 in this study) suggests that IKAP also has biological functions outside the

nucleus. Yet, IKAP is the closest human homologue of the *S. cerevisiae* protein Elp1/IKI3 (29% shared identity), the largest subunit of the yeast elongator. The transcriptional elongator function of Elp1 has been rendered probable genetically, as *elp1* deleted (*elp1D*) yeast cells co-depleted for TFIIS are synthetically hypersensitive to 6-azauracil (3). We note however, that many of the described *elp1D* phenotypes are equally consistent with delayed stress responses.

IKAP is well conserved in the eukaryotic kingdom. When aligned, the *Saccharomyces*, *Arabidopsis*, and *Drosophila* orthologues show extensive identity over their entire lengths. Sequence analysis predicts the N-terminal half of IKAP to contain seven successive WD40-like repeats. WD40 domains exist in numerous eukaryotic proteins that cover a wide variety of adaptor/regulatory functions in signal transduction, pre-mRNA processing, and cytoskeletal assembly. Importantly, the WD40 motifs do not possess any catalytic activity but are rather involved in protein binding (56). Most of the C-terminal half of IKAP is predicted to contain TPR-like motifs. TPR motifs are presumed to form helix-turn-helix structures and are found in diverse groups of protein involved in various cellular processes including mitosis, transcription, splicing, protein import, and neurogenesis. Like WD40, TPR repeats are believed to mediate protein-protein interactions (57).

The WD40- and TPR-like domains in IKAP are separated with a linker region that does not exhibit any known domain structure. Interestingly, the JNK binding site in IKAP also resides outside these two major IKAP domain structures. The predicted domain structure of IKAP suggests that this large protein may be involved in various cellular processes and is likely to form multiprotein complexes within the cell. Indeed, our results demonstrated a role of IKAP in JNK activation, whereas work from Svejstrup and co-workers (4) links IKAP to transcriptional elongation.

The Model for the Involvement of IKAP in the JNK Pathway—Various independent studies have been carried out to understand how MAPKKKs mediate their effector functions through phosphorylation of MAPKKs and MAPKs. These studies have demonstrated that some of the MAPK modules are assembled via scaffolding or anchoring proteins to obtain the signaling specificity. In the yeast mating response, Ste5 serves as a scaffold protein that brings together the MAPKKK Ste11, MAPKK Ste7, and MAPK Fus3, allowing them to activate each other (for a recent review, see Ref. 58). In mammalian cells, JIP-1 has been described as a "functional homologue of Ste5," a scaffold protein involved in the assembly of the mammalian MAPK module MLK/MKK7/JNK via direct interaction with all three proteins (23, 59).

A similar role is recently suggested for JSAP-1, which was shown to form the MEKK1/JNKK1/JNK3 module via direct interaction with MEKK1 and JNK3 (40) and β-arrestin in forming the ASK1/JNKK/JNK3 module (41). The activation of JNK by co-expression with MEKK1 and/or ASK1 and IKAP resembles the activation of JNK induced by JIP-1, JSAP-1, and β-arrestin 2 (39–41). Our results hint that IKAP could be involved in assembly of JNK-MAPK module. In this study we show that IKAP associates with JNK and facilitates its activation. Despite apparent similarities, the cooperation of JNK with IKAP has also clear differences from what has been described for the other scaffold proteins. Most importantly, we were not able to detect any clear and strong interactions between IKAP and MEKK1, ASK1, JNKK1, or JNKK2 (data not shown). Furthermore JIP-1, JSAP-1 (JIP-3), and β-arrestin 2 seem to be very selective with their MAPKKK component, whereas IKAP works with both MEKK1 and ASK1. Thus, according to present study, the impact of IKAP on the JNK pathway seems to involve different mechanisms than those of the previously reported JNK scaffold proteins.

Role of JNK in FD—The developmental- and stress-induced neuronal phenotypes of mice lacking functional JNK1, -2, or -3 genes and the JNK1, -2, and -3 compound mutant mice have been widely reported. They demonstrate both protective and promotive roles of JNK in neuronal apoptosis (for review, see Ref. 12). JNK activation is also known to be part of the neuronal stress response after neuronal injury and degeneration as well as regeneration (60, 61). Two recent studies demonstrate an IKAP splicing mutation as a cause of FD, an autosomal recessive disorder characterized by poor development and survival combined with progressive degeneration of the sensory and autonomic nervous system (5, 6). This IKAP-splicing mutation can cause skipping of exon 20 in the mRNA of the patients with FD and results in expression of a shorter form of IKAP truncated at amino acid 714, here referred to as FD-IKAP (see Fig. 5*B*). However, the penetrance of the mal-splicing is not complete, since patients with FD sustain expression of various levels of wild type message in a tissue-specific manner (6).

It was suggested earlier that FD might be caused by a deficiency in neuronal growth factor pathway (62, 63). Neuronal growth factor deprivation from the cultured sympathetic neurons will lead to JNK activation and cell death. Neuronal growth factor-deprived neurons can be rescued either by the addition of neuronal growth factor or by inhibition of JNK (64, 65). Interestingly, our study shows that the region deleted from FD-IKAP contains the site responsible for JNK association and activation. As such, the FD-IKAP protein is unable to regulate JNK activation, and we hypothesize that neuronal degeneration in FD patients may be caused by improperly regulated stress pathways, in particular the JNK pathway. In addition, IKAP may serve important functions in transcriptional elongation. We speculate that the elongator functions of IKAP/Elp1 could be particularly important for expression of stress-inducible genes. Indeed, it is possible that IKAP is involved first with activation of the stress kinase and then with efficient stress gene expression.

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REFERENCES

1. Cohen, L., Henzel, W. J., and Baeuerle, P. A. (1998) *Nature* **395,** 292–296
2. Krappmann, D., Hatada, E. N., Tegethoff, S., Li, J., Klippel, A., Giese, K., Baeuerle, P. A., and Scheidereit, C. (2000) *J. Biol. Chem.* **275,** 29779–29787
3. Otero, G., Fellows, J., Li, Y., de Bizemont, T., Dirac, A. M., Gustafsson, C. M., Erdjument-Bromage, H., Tempst, P., and Svejstrup, J. Q. (1999) *Mol. Cell* **3,** 109–118
4. Hawkes, N. A., Otero, G., Winkler, G. S., Marshall, N., Dahmus, M. E., Krappmann, D., Scheidereit, C., Thomas, C. L., Schiavo, G., Erdjument-Bromage, H., Tempst, P., and Svejstrup, J. Q. (2002) *J. Biol. Chem.* **277,** 3047–3052
5. Anderson, S. L., Coli, R., Daly, I. W., Kichula, E. A., Rork, M. J., Volpi, S. A., Ekstein, J., and Rubin, B. Y. (2001) *Am. J. Hum. Genet.* **68,** 753–758
6. Slaugenhaupt, S. A., Blumenfeld, A., Gill, S. P., Leyne, M., Mull, J., Cuajungco, M. P., Liebert, C. B., Chadwick, B., Idelson, M., Reznik, L., Robbins, C., Makalowska, I., Brownstein, M., Krappmann, D., Scheidereit, C., Maayan, C., Axelrod, F. B., and Gusella, J. F. (2001) *Am. J. Hum. Genet.* **68,** 598–605
7. Takeoka, S., Unoki, M., Onouchi, Y., Doi, S., Fujiwara, H., Miyatake, A., Fujita, K., Inoue, I., Nakamura, Y., and Tamari, M. (2001) *J. Hum. Genet.* **46,** 57–63
8. Ip, Y. T., and Davis, R. J. (1998) *Curr. Opin. Cell Biol.* **10,** 205–219
9. Leppa, S., and Bohmann, D. (1999) *Oncogene* **18,** 6158–6162
10. Gupta, S., Campbell, D., Derijard, B., and Davis, R. J. (1995) *Science* **267,** 389–393
11. Mohit, A. A., Martin, J. H., and Miller, C. A. (1995) *Neuron* **14,** 67–78
12. Davis, R. J. (2000) *Cell* **103,** 239–252
13. Yang, D. D., Kuan, C. Y., Whitmarsh, A. J., Rincon, M., Zheng, T. S., Davis, R. J., Rakic, P., and Flavell, R. A. (1997) *Nature* **389,** 865–870
14. Behrens, A., Sibilia, M., and Wagner, E. F. (1999) *Nat. Genet.* **21,** 326–329
15. Kuan, C. Y., Yang, D. D., Samanta Roy, D. R., Davis, R. J., Rakic, P., and Flavell, R. A. (1999) *Neuron* **22,** 667–676

16. Sabapathy, K., Jochum, W., Hochedlinger, K., Chang, L., Karin, M., and Wagner, E. F. (1999) *Mech. Dev.* **89,** 115–124
17. Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994) *Cell* **76,** 1025–1037
18. Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) *Genes Dev.* **7,** 2135–2148
19. Kallunki, T., Su, B., Tsigelny, I., Sluss, H. K., Derijard, B., Moore, G., Davis, R., and Karin, M. (1994) *Genes Dev.* **8,** 2996–3007
20. Sluss, H. K., Barrett, T., Derijard, B., and Davis, R. J. (1994) *Mol. Cell. Biol.* **14,** 8376–8384
21. Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J., and Woodgett, J. R. (1994) *Nature* **369,** 156–160
22. Garrington, T. P., and Johnson, G. L. (1999) *Curr. Opin. Cell Biol.* **11,** 211–218
23. Whitmarsh, A. J., and Davis, R. J. (1998) *Trends Biochem. Sci.* **23,** 481–485
24. Blank, J. L., Gerwins, P., Elliott, E. M., Sather, S., and Johnson, G. L. (1996) *J. Biol. Chem.* **271,** 5361–5368
25. Gerwins, P., Blank, J. L., and Johnson, G. L. (1997) *J. Biol. Chem.* **272,** 8288–8295
26. Lange-Carter, C. A., Pleiman, C. M., Gardner, A. M., Blumer, K. J., and Johnson, G. L. (1993) *Science* **260,** 315–319
27. Lewis, T. S., Shapiro, P. S., and Ahn, N. G. (1998) *Adv. Cancer Res.* **74,** 49–139
28. Derijard, B., Raingeaud, J., Barrett, T., Wu, I. H., Han, J., Ulevitch, R. J., and Davis, R. J. (1995) *Science* **267,** 682–685
29. Lin, A., Minden, A., Martinetto, H., Claret, F. X., Lange-Carter, C., Mercurio, F., Johnson, G. L., and Karin, M. (1995) *Science* **268,** 286–290
30. Sanchez, I., Hughes, R. T., Mayer, B. J., Yee, K., Woodgett, J. R., Avruch, J., Kyriakis, J. M., and Zon, L. I. (1994) *Nature* **372,** 794–798
31. Yan, M., Dai, T., Deak, J. C., Kyriakis, J. M., Zon, L. I., Woodgett, J. R., and Templeton, D. J. (1994) *Nature* **372,** 798–800
32. Kallunki, T., Deng, T., Hibi, M., and Karin, M. (1996) *Cell* **87,** 929–939
33. Karin, M., Liu, Z., and Zandi, E. (1997) *Curr. Opin. Cell Biol.* **9,** 240–246
34. Buschmann, T., Adler, V., Matusevich, E., Fuchs, S. Y., and Ronai, Z. (2000) *Cancer Res.* **60,** 896–900
35. Chauhan, D., Hideshima, T., Treon, S., Teoh, G., Raje, N., Yoshihimito, S., Tai, Y. T., Li, W., Fan, J., DeCaprio, J., and Anderson, K. C. (1999) *Cancer Res.* **59,** 1192–1195
36. Gupta, S., Barrett, T., Whitmarsh, A. J., Cavanagh, J., Sluss, H. K., Derijard, B., and Davis, R. J. (1996) *EMBO J.* **15,** 2760–2770
37. Kharbanda, S., Saxena, S., Yoshida, K., Pandey, P., Kaneki, M., Wang, Q., Cheng, K., Chen, Y. N., Campbell, A., Sudha, T., Yuan, Z. M., Narula, J., Weichselbaum, R., Nalin, C., and Kufe, D. (2000) *J. Biol. Chem.* **275,** 322–327
38. Dickens, M., Rogers, J. S., Cavanagh, J., Raitano, A., Xia, Z., Halpern, J. R., Greenberg, M. E., Sawyers, C. L., and Davis, R. J. (1997) *Science* **277,** 693–696
39. Whitmarsh, A. J., Cavanagh, J., Tournier, C., Yasuda, J., and Davis, R. J. (1998) *Science* **281,** 1671–1674
40. Ito, M., Yoshioka, K., Akechi, M., Yamashita, S., Takamatsu, N., Sugiyama, K., Hibi, M., Nakabeppu, Y., Shiba, T., and Yamamoto, K. I. (1999) *Mol. Cell. Biol.* **19,** 7539–7548
41. McDonald, P. H., Chow, C. W., Miller, W. E., Laporte, S. A., Field, M. E., Lin, F. T., Davis, R. J., and Lefkowitz, R. J. (2000) *Science* **290,** 1574–1577
42. Cheng, J., Yang, J., Xia, Y., Karin, M., and Su, B. (2000) *Mol. Cell. Biol.* **20,** 2334–2342
43. Xia, Y., Wu, Z., Su, B., Murray, B., and Karin, M. (1998) *Genes Dev.* **12,** 3369–3381
44. Xu, S., and Cobb, M. H. (1997) *J. Biol. Chem.* **272,** 32056–32060
45. Park, H. S., Lee, J. S., Huh, S. H., Seo, J. S., and Choi, E. J. (2001) *EMBO J.* **20,** 446–456
46. Shim, J., Lee, H., Park, J., Kim, H., and Choi, E. J. (1996) *Nature* **381,** 804–806
47. Shim, J., Park, H. S., Kim, M. J., Park, J., Park, E., Cho, S. G., Eom, S. J., Lee, H. W., Joe, C. O., and Choi, E. J. (2000) *J. Biol. Chem.* **275,** 14107–14111
48. Aronheim, A. (2001) *Methods Enzymol.* **332,** 260–270
49. Broder, Y. C., Katz, S., and Aronheim, A. (1998) *Curr. Biol.* **8,** 1121–1124
50. Legerski, R., and Peterson, C. (1992) *Nature* **359,** 70–73
51. Zandi, E., Rothwarf, D. M., Delhase, M., Hayakawa, M., and Karin, M. (1997) *Cell* **91,** 243–252
52. Han, J., Jiang, Y., Li, Z., Kravchenko, V. V., and Ulevitch, R. J. (1997) *Nature* **386,** 296–299
53. Aronheim, A., Zandi, E., Hennemann, H., Elledge, S. J., and Karin, M. (1997) *Mol. Cell. Biol.* **17,** 3094–3102
54. Minden, A., Lin, A., Smeal, T., Derijard, B., Cobb, M., Davis, R., and Karin, M. (1994) *Mol. Cell. Biol.* **14,** 6683–6688
55. Karin, M. (1999) *Oncogene* **18,** 6867–6874
56. Garcia-Higuera, I., Fenoglio, J., Li, Y., Lewis, C., Panchenko, M. P., Reiner, O., Smith, T. F., and Neer, E. J. (1996) *Biochemistry* **35,** 13985–13994
57. Goebl, M., and Yanagida, M. (1991) *Trends Biochem. Sci.* **16,** 173–177
58. Burack, W. R., and Shaw, A. S. (2000) *Curr. Opin. Cell Biol.* **12,** 211–216
59. Yasuda, J., Whitmarsh, A. J., Cavanagh, J., Sharma, M., and Davis, R. J. (1999) *Mol. Cell. Biol.* **19,** 7245–7254
60. Herdegen, T., Claret, F. X., Kallunki, T., Martin-Villalba, A., Winter, C., Hunter, T., and Karin, M. (1998) *J. Neurosci.* **18,** 5124–5135
61. Kenney, A. M., and Kocsis, J. D. (1998) *J. Neurosci.* **18,** 1318–1328
62. Breakefield, X. O., Orloff, G., Castiglione, C., Coussens, L., Axelrod, F. B., and Ullrich, A. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 4213–4216
63. Breakefield, X. O., Ozelius, L., Bothwell, M. A., Chao, M. V., Axelrod, F., Kramer, P. L., Kidd, K. K., Lanahan, A. A., Johnson, D. E., Ross, A. H., *et al.* (1986) *Mol. Biol. Med.* **3,** 483–494
64. Eilers, A., Whitfield, J., Shah, B., Spadoni, C., Desmond, H., and Ham, J. (2001) *J. Neurochem.* **76,** 1439–1454
65. Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J., and Greenberg, M. E. (1995) *Science* **270,** 1326–1331