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.

IKAP1 was originally purified as a 150-kDa-protein component of the large interleukin 1-inducible IκB complex containing NIK, IκKα, IκKβ, and NF-κB/RelA and suggested to be a scaffold protein involved in the assembly of the IκB kinase (IKK) complex (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 IκB complexes.

Instead, it was suggested that IKAP is involved in general 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 homozygosity 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 kinase acid-induced excitotoxicity, resulting in decreased apoptosis in the hippocampus, 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-al/ala knockout 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 corticofugal 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)-signal transduction, 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 (MAPKKKK), a MAP kinase kinase (MAPKKK), 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 MAPKKks 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-associating 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 JNK1 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 acids 140–1333) 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, IKAP, 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 XhoI site and reverse-transcribed, and the resulting cDNA was ligated to EcoRI linkers. The cDNA was digested with EcoRI and XhoI and inserted into pYesMΔ poly(A) plasmid (49). The library consisted of 4 million independent transformants. The deletion mutants of 102-IKAP, 102ΔC-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 pYesMΔ plasmid (49). The library consisted of 4 million independent transformants. The deletion mutants of 102-IKAP, 102ΔC-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 (JNK1ΔMK, 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 XmaI and HindIII 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 HisXpress tag at BamHI and NotI 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-Not1 (TCC TAA CTG 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 XbaI restriction site (located in the Myc sequence (1), so that both PCR products would together contain the full open reading frame of IKAP. A HeLa cell cDNA library was used as a template. The obtained fragments were directly subcloned into the pXMyc expression vector between BamHI and NotI restriction sites. All 10 individual clones were sequenced, and 1 of the clones, pXMyc6, 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 BamHI site to the reading frame of the His-Xpress tag of pcDNA3.1His. The PCR product was digested with BamHI/XbaI and XbaI/NotI, and the two fragments were cloned into pcDNA3.1His between BamHI and NotI sites. This PCR strategy removed the first eight codons of the IKAP reading frame. Primer sequences were as follows: upstream primer 5' - GTCCGATCCGGAGTTCAGGGATATTCA-3' and downstream primer (5' - TCC TAA CTG CCG CTC AGT CTA GCA GG-3'). The IKAP C-terminal deletion construct pcDNA3.1His-IKAP4C was also made by a PCR-based strategy. The pcDNA3.1His-IKAP2C plasmid was made by PCR using oligos Bam-IK2 (5' - CGG GAT CCA TGA ACT TTA CCA AAG-3') and IK-NotI 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 NcoI site in IKAP, and thus, it contains amino acids 1070–1333 of IKAP fused in-frame with the HA tag of pSRo3, analogous to pSRo3-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-Xpress tagged IKAP cDNA from pcDNA3.1His-IKAP using primers 5' - GTCGACCAAGGCTAGCTCAGTTAAC-3' and 5' - TAGATCTACGGGCTACAGTCCACTGG-3' and inserted it as a SalI/BglII restriction fragment between the XhoI and BamHI 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-JNK1 wild type and dominant negative, APF-JNK, 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 transfection protocol using the lipofectamine reagent (Molecular Probes) as medium. HeLa cells expressing the IKAP/green fluorescent protein fusion were fixed in 4% paraformaldehyde before washing. Fluorescence micrographs 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 ml Trits, pH 7.6, 100 mM sodium chloride, 1 mM EDTA, 1 mM sodium vanadate, 15 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 ml 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 radioimmuno 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 μl of diluted substrates, 1 μM ATP, and 5 μCi of [γ-32P]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 constructs designed for a catalytically inactive JNK fusion (IKAP-KM) fused to N. Ras (IKAP-Dn.Ras(61A,F)) were screened about 10% of the myristoylated expression library (53) made from mRNA isolated from TNF-α and interleukin 1-stimulated HeLa cells. Five individual clones exhibited efficient growth at the restrictive temperature when grown on galactose-containing medium. DNA extracted from these clones was reintroduced
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 or JNK1-Wt.Ras(61)/H9004F alone was observed (Fig. 1A, lane 2). 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 anti-IKAP 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. 1B; 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 and autoradiographed, IKAP 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.

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)/H9004F), JNK1 wild type bait (JNK1-Wt.Ras(61)/H9004F) or inactive JNK1 bait (JNK1-Dn.Ras(61)/H9004F). 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-32P]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.
IKAP Regulates JNK Activation

As evident from Fig. 2A, 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.

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.

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. 2A, 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. 2B). Overlay of the two stains revealed partial co-localization (Fig. 2C) 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. 2E). A similar pattern was also observed using ectopically expressed green fluorescent protein-tagged IKAP (Fig. 2F). 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 32P-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. 2E) in the absence or presence of increasing concentrations of MEKK1, a most potent activator of JNK (54). JNK activation was measured by immunocomplex kinase assay using GST-ΔJun (1–79) as a substrate. The overexpression of IKAP alone moderately activated JNK (Fig. 4A; compare lanes 1 and 5). Overexpression of IKAP together with low concentrations of MEKK1 resulted in further potentiation of JNK1 activity (Fig. 4A; 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. 4A; 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 MEKK1-induced JNK activation was found highly significant (p = 0.0018) in Student’s t test (Fig. 4B).
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-IKK/H9252 together with increasing concentrations of MEKK1/H9004 (Fig. 4C; top panel, lanes 1–4) did not result in any measurable effect on the activity of IKK/H9252 as compared with the vector control (Fig. 4C; top panel, lanes 5–8). Again, ectopic expression of HA-IKK/H9252 was not affected by IKAP (Fig. 4C; lower panel).

We also tested the ability of IKAP to facilitate ASK1-induced JNK activation in a similar experimental setting (Fig. 4D). Co-expression of the His/X-press IKAP with the increasing concentrations of ASK1 (Fig. 4D; top panel, lanes 1–4) clearly potentiated JNK activity compared with the vector control (Fig. 4D; top panel, lanes 5–8). The quantifications represent the means of three independent experiments. The expression of FLAG-JNK1 was unaffected by IKAP (Fig. 4D, lower panel).

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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. 4F, lanes 1–4) or absence of IKAP (Fig. 4F, lanes 5–8). Cells were treated with the indicated doses of the known JNK activators, TNF, epidermal growth factor, or 100 J/m² 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. 4F; 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, IKKa, 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. 5A; 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. 5A, 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 coprecipitation method. JNK was precipitated (IP) with HA antibody, and the immunocomplex kinase assays were performed using GST-c-Jun.
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 IKKa, 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

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.

(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 C 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-IκB as a substrate. Expression of the HA-IKKβ in each individual case is determined by Western blotting (lower panel). D, IKAP potentiates ASK1 induced JNK activity. 293 cells were co-transfected with 6 μg of FLAG-tagged IKAP 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-Gl-32P]ATP was quantified using phosphorimaging, and the expression of FLAG–JNK1 was determined 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-IKAP 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/m² of UV light. Incorporation of the [γ-32P]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. 5B). 293 cells were co-transfected together with HA-JNK1 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. 6A). 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. 6A). 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 transfection, JNK activity was induced with co-expression of MEKK1Δ (Fig. 6B; lanes 2 and 4), and in the other two, activity was induced with UV-C irradiation (Fig. 6B; 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. 6B; compare lanes 2–4 and lanes 6–8).

**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 IKAP 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, however, it 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. 5B).

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 po-

**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-IKKs 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/m2) 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.
IKAP Regulates JNK Activation

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 MAPKKs mediate their effector functions through phosphorylation of MAPKKs and MAPKs. These studies have demonstrated that some of the MAPK modules are believed to mediate protein-protein interactions (57).

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 MEK1 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, JNK1, or JNK2 (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 that 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. 5B). 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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