Human JIK, a Novel Member of the STE20 Kinase Family That Inhibits JNK and Is Negatively Regulated by Epidermal Growth Factor*

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Mammalian members related to Saccharomyces cerevisiae serine/threonine kinase STE20 can be divided into two subfamilies based on their structure and function. The PAK subfamily is characterized by an N-terminal p21-binding domain (also known as CRIB domain), a C-terminal kinase domain, and is regulated by the small GTP-binding proteins Rac1 and Cdc42Hs. The second group is represented by the GCK-like members, which contain an N-terminal catalytic domain and lack the p21-binding domain. Some of them have been demonstrated to induce c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) cascade, while others have been shown to be activated by a subset of stress conditions or apoptotic agents, although little is known about their specific function. Here, we have identified a novel human STE20-related serine/threonine kinase, belonging to the GCK-like subfamily. This kinase does not induce the JNK/SAPK pathway, but, instead, inhibits the basal activity of JNK/SAPK, and diminishes its activation in response to human epidermal growth factor (EGF). Therefore, we designated this molecule JIK for JNK/SAPK-inhibitory kinase. The inhibition of JNK/SAPK signaling pathway by JIK was found to occur between the EGF receptor and the small GTP-binding proteins Rac1 and Cdc42Hs. In contrast, JIK does not activate nor does it inhibit ERK2, ERK6, p38, or ERK5. Furthermore, JIK kinase activation is not modulated by any exogenous stimuli, but, interestingly, it is dramatically decreased upon EGF receptor activation. Thus, JIK might represent the first member of the STE20 kinase family whose activity can be negatively regulated by tyrosine kinase receptors, and whose downstream targets inhibit, rather than enhance, JNK/SAPK activation.

Binding of growth factors, hormones, and cytokines to their receptors regulates intracellular signal transduction pathways, which, in turn, control diverse cellular functions such as metabolism, proliferation, differentiation, programmed cell death, and stress responses. The biochemical routes mediating these diverse biological responses often involve a series of sequentially organized serine-threonine protein kinases, known collectively as mitogen-activated protein kinase (MAPK) cascades (1–3). In mammalian cells, these kinases include p44/42 MAPK also known, respectively, as extracellular signal-regulated kinases 1 and 2 (ERK-1 and ERK-2) (4, 5), ERK5 (6), ERK6, also known as SAPK3 (7), c-Jun N-terminal kinases/stress-activated protein kinase (JNK/SAPK) (8–11), p38 kinase (11, 12), and SAPK4 (13).

The best studied MAPKs are the ERKs, whose catalytic activity is elevated in response to the binding of ligands to receptor tyrosine kinases (i.e. epidermal growth factor receptors) or G protein-coupled receptors. The activation occurs after phosphorylation catalyzed by their upstream protein kinase known as MAPK kinase (MEK) (14–16), which is itself activated by phosphorylation on two serine residues by several mammalian serine/threonine kinases, including Raf (17–19), Mos (20), and MEK1 kinase (MEKK1) (21). In contrast, JNKs/SAPKs are activated in response to stress-inducing signals, such as osmotic and heat shock, UV light, protein synthesis inhibitors, and proinflammatory cytokines (8–10). It has been recently shown that MKK4/SAPK/JNK/SEK1 phosphorylates JNK/SAPK at threonine and tyrosine, thus causing its activation (22–24). SEK1 is regulated by phosphorylation by upstream MAPK kinase kinases, which include MEKK1, -2, -3, and -4 (21, 25–28), Tpl-2 (29), and MAPKKK5 (30). However, upstream molecules controlling MEKK1 directly are currently unknown.

The function of p38 in mammalian cells is unclear, but recent data indicate that it may respond to proinflammatory cytokines (interleukin-1, TNF-α, lipopolysaccharide) and environmental stress (osmotic shock) (12, 30–33). Pathways regulating the activity of ERK6 (34), and SAPK4 appear to be similar to those of p38 (13), while ERK5, shown to be activated by oxidative stress, may represent a unique redox-sensitive kinase distinct from other MAPK family members (35, 36).

This unexpected diversity of MAPKs is not unique to mammalian cells. In the budding yeast Saccharomyces cerevisiae, at least six MAPK pathways have been identified (37, 38). They regulate diverse biological processes, such as mating and inva-

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1 The abbreviations used are: MAPK, mitogen-activated protein kinase; HA, hemagglutinin; PAG, polyacrylamide gel electrophoresis; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; JNK, c-Jun N-terminal kinase; MBP, myelin basic protein; PBS, phosphate-buffered saline; TNF-α, tumor necrosis factor-α; DTT, dithiothreitol; GST, glutathione S-transferase; MEK, mitogen-activated protein kinase kinase; MEKK, mitogen-activated protein kinase kinase kinase; ERK, extracellular signal-regulated kinase; SAPK, stress-activated protein kinase; JIK, JNK/SAPK-inhibitory kinase.
sive growth, cell wall integrity, and the response to high osmolarity (39–42). Based on epistasis experiments, the S. cerevisiae serine-threonine kinase STE20 was shown to act upstream of the MAPK module consisting of the MAPK kinase kinase STE11, the MAPK kinase STE7, and the MAPKs FUS3/KSS1 in the mating pathway (43–45). Additional S. cerevisiae STE20-like kinases have been identified, and include Cla4 (46), which is involved in budding and cytokinesis, and SPS1 (47), a kinase required for late events in sporulation. In fission yeast, the STE20-like kinase Shk1 forms a complex with Cdc42 and a functional interaction between Shk1, Cdc42, and Ras1 is required for normal cell morphology and mating (48).

In the last few years, a large number of mammalian kinases closely related to S. cerevisiae STE20 have been identified. Based on their structure, these mammalian STE20-like kinases can be divided into two subfamilies (49): the PAK subfamily, containing a N-terminal p21-binding domain (also known as CRIK domain) and a C-terminal kinase domain; and the GCK-like subfamily, which contains a N-terminal catalytic domain and lacks the p21-binding domain. Recently, it has been shown that PAK comprises a protein kinase family composed of several PAK isoforms, including the rat and mouse p21-activated protein kinases, p65AKAP and mPAK-3 (50, 51), and their human counterparts, hPAK65 (hPAK2) (52, 53) and hPAK1 (53, 54), which are all able to interact with the small p21 GTP-binding proteins Rac1 and Cdc42 (50), thereby regulating the activation of JNK/SAPK and p38 pathways (55–57).

The second group of kinases comprises a growing number of serine/threonine kinases, including nNik (58) (Nck-interacting kinase), hKHS (59) (kinase homologous to SPS1/STE20), hGCK (60) (GCK-like kinase), hHPK1 (62) and mHPK1 (63) (hematopoietic progenitor kinase-1), hSOK-1 (64) (STE20/oxidant stress response kinase-1), mLOK (65) (lymphocyte-cytied kinase), hMST1 (66) (mammalian STE20-like kinase-1), hMST2 (67) (mammalian STE20-like kinase-2) and hMST3 (68) (ammmalian E20-like kinase-3), and hKR51 and 2 (69) (kinases responsive to gression). In this study, we describe the isolation of a novel human serine/threonine kinase, designated JNK/SAPK-inhibitory kinase (JIK), whose kinase domain shares similarity to the GCK-like subfamily of STE20 kinases. Furthermore, its non-catalytic domain shares high homology to Caenorhabditis elegans putative serine/threonine kinase, named SULU, of unknown function. Interestingly, we found that this protein does not induce JNK/SAPK pathway; rather, its overexpression in COS7 cells leads to the inhibition of JNK/SAPK activation. Furthermore, EGFR negatively regulates JIK activity, and thus it may represent the first member of the STE20-like kinases whose activity is reduced upon stimulation of a cell surface receptor.

**MATERIALS AND METHODS**

**Isolation and Characterization of JIK cDNA**—A GST fusion protein containing eps8-SH3 domain (amino acid residues 531–591) was used to screen a pCEV-LAC bacterial expression library constructed from human embryonic fibroblast M426 mRNAs, as described previously (70). The library was a kind gift from Dr. Toru Miki (71).

Northern Blot Analysis—DNA probes used for Northern blot analysis were labeled with α-32PdCTP using a random priming kit (Stratagene). The JIK cDNA (nucleotides 1100–2800) and the human glycerolaldehyde 3-phosphate dehydrogenase were random primed (Stratagene), and Northern blot analysis was performed on commercial human multiple tissue Northern blots (CLONTECH). Hybridization was performed as recommended by the manufacturer. **Production of Anti-JIK Polyclonal Antibodies**—Two oligonucleotide primers, 5′-CCCGGATCCAGATGAGATGAAGCCGATTCGCG-3′ and 5′-TGAATATCATCTTTCATGTGCTCCTTCTGAAAATC-3′, corresponding respectively, to amino acids 461–469 and 891–898, were used to amplify the cDNA from the non-catalytic region of JIK (clone 74). The resulting fragment was cloned in frame into the BamHI and HindIII sites of pGEX-KG vector (Amersham Pharmacia Biotech). GST-JIK fusion protein was purified as described (72) and used to raise polyclonal antibodies in rabbit.

**Expression Plasmids**—BamHI-EcoRI cDNA fragments containing either the entire coding sequence of JIK or JIK-A181 (E183) (73) were amplified and introduced into BamHI-EcoRI sites of pCEFL-HA (74) or pCEFL-AU5 vectors. pCEFL-HA was transfected into the DNAH11 expression vectors containing the elongation factor-1 promoter driving the expression of an in-frame N-terminal tag of nine amino acids (YYPDYVDPDA) or six amino acids (TDYFKL) derived from HA and AU5, respectively.

Mutations were introduced into pBluescriptII SK-JIK by polymerase chain reaction (QuickChange mutagenesis kit; Stratagene) according to the manufacturer’s instructions and subcloned into the BamHI-EcoRI sites of pCEFL-HA and pCEFL-AU5 expression vectors. The expression plasmids were sequenced by DDBJ.

**Western Blot Analysis**—pCEFL-HA-JIK, pCEFL-HA-JIK-K, or pCEFL-HA-JIK-A181 (E183) were transfected into human C33A and COS7 cells by calcium phosphate and DEAE-dextran methods, respectively.

**Phosphorylation and Immunoprecipitation Assays**—Subconfluent C33A cells were transfected with 10 μg of pCEFL-HA, pCEFL-HA-JIK, or pCEFL-HA-JIK-A181 (E183). Two days after transfection, the cells were washed with cold PBS and lysed at 4°C in a buffer containing 20 mM Hepes (pH 7.5), 10 mM EGTA (pH 8), 2.5 mM MgCl2, 1 mM DTT, 1% Nonidet P-40, 0.1% sodium deoxycholate, 1% sodium dodecyl sulfate, and 20 μg/ml leupeptin. The immunoprecipitation procedure and the subsequent kinase assay were performed as described (55). Briefly, the epitope-tagged JIK and JIK-A181 (E183) were respectively immunoprecipitated from the cleared lysates by incubation with anti-HA (12CA5) for 1 h at 4°C. Immunocomplexes were recovered with the aid of Gamma-Bind Sepharose beads (Amersham Pharmacia Biotech) and washed three times with PBS containing 1% Nonidet P-40 and 1 mM sodium otovanadate, once with 100 mM Tris (pH 7.5) and 0.5 mM CaCl2 and once with kinase reaction buffer (25 mM Hepes, pH 7.5, 20 mM MgCl2, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2 mM DTT). JIK activity was determined by resuspension in 30 μl of kinase reaction buffer containing 1 μCi (4500 Ci/mmol) of [γ-32P]ATP per reaction, 20 μM of cold ATP, and 4 μg of myelin basic protein as substrate. After incubation for 30°C for 20 min, reactions were terminated by the addition of 15 μl of 5× Laemmli’s buffer. Samples were heated at 95°C for 5 min, analyzed by SDS electrophoresis on 12.5% polyacrylamide gels, and visualized by autoradiography. Parallel anti-HA immunoprecipitates were processed for Western blot analysis using an anti-JIK-specific antisera.

**MAPK Assays**—Subconfluent COS7 cells were transfected with 1 μg of pCDNA3-HA-ERK2, pCEFL-HA-ERK5, pCEFL-HA-ERK6, pCEFL-HA-SAPK4, pCEFL-AU5-Rac1QL, pCEFL-AU5-Cdc4QQL, pCEFL-AU5-MLK3, and pCEV29-MEK1 have been already described (55, 73).

**Cell Cultures and Transfections**—C33A and COS7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum. NIH-3T3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) calf serum. Transfection of C33A and COS7 cells was performed by calcium phosphate and DEAE-dextran methods, respectively.

**Phosphoamino acid analysis**—Phosphoamino acids were identified by autoradiography. 2-Dimensional gel electrophoresis was performed as described (72) and the spots corresponding to myelin basic protein were excised and hydrolyzed with 6N HCl at 110°C for 90 min. The hydrolyzed sample was diluted in 1 ml of water, frozen in dry ice, dried by centrifugation under vacuum, and resuspended in 10 μl of water containing 2 mg/ml phosphotyrosine, phosphoserine, and phosphothreonine standard, respectively. 1 μl of the sample was then spotted onto a cellulose thin layer chromatography plate. Electrophoresis was performed in a buffer containing 1% pyridine and 10% acetic acid at 800 V for 75 min. The plates were then air-dried, and the amino acids were visualized with 0.25% ninhydrin in ace tone. Phosphoamino acids were identified by autoradiography.
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Immunoblot Analysis—Anti-HA, anti-AU5, or anti-JIK immunoprecipitates were analyzed by Western blotting after SDS-PAGE, transferred to Immobilon P membranes (Millipore), and immunoblotted with the corresponding rabbit antiserum or mouse monoclonal antibody. Immunocomplexes were visualized by enhanced fluoroluminescence detection (Vistra ECF, Amersham Pharmacia Biotech) using alkaline phosphatase-linked goat anti-rabbit or anti-mouse antiserum. Mouse monoclonal antibodies, anti-HA (clone 12CA5) and anti-AU5 were purchased from BABCO. Rabbit polyclonal anti-JNK1, anti-ERK2, and anti-p38 were purchased from Santa Cruz Biotechnology, Inc. Anti-ERK5 was purchased from Stressgen.

RESULTS

Eps8 is an SH3 domain-containing protein identified as a substrate for the epidermal growth factor receptor (EGFR) and several other receptor tyrosine kinases (76). The binding site of eps8 on the EGFR was mapped to the juxtamembrane region of this receptor (77). Although several observations suggest that eps8 plays a critical role in mitogenic signaling (76, 78), its cellular function is still not clearly understood.

As an approach to identify proteins that can associate physically with eps8, we screened a human embryonic fibroblast M426 expression library using the GST SH3 domain of eps8 as a probe (70). Several overlapping cDNA clones were identified, the longest of which, designated clone 74–10, contained a cDNA insert of 2,978 kilobase pairs. The complete nucleotide sequence of the clone 74–10 (GenBank accession no. AF179867) predicted an 898-amino acid open reading frame (Fig. 1A) and a molecular mass of 106 kDa. Furthermore, the clone 74 contained a consensus Kozak sequence, GCCATC (79), immediately upstream from the putative initiation codon. GenBank and EMBL data base searches revealed that its N-terminal region (amino acids 19–284) encompasses a kinase domain including all 11 subdomains that are characteristic of serine/threonine kinases (80). Sequence comparison with other protein kinases using the BLAST program (81) demonstrated that the kinase domain of clone 74 shares sequence homology with members of the STE20-like kinase family, and is most closely related to MST1 and MST2 (57% identity) (66, 67), SOK1 (56% identity) (64), SLK (51% identity) (82), hPAK1 (47% identity) (53, 54), KHS1 (45% identity) (59), and NIK (44% identity) (58). Furthermore, the overall protein exhibits 63% identity and 76% similarity with a C. elegans serine/threonine kinase of unknown function, designated SULU (Fig. 1B). A dendrogram was created to examine the evolutionary relationship between clone 74, designated JIK for Jun kinase-inhibitory kinase (see below), and the other members of the STE20-like kinases. As shown in Fig. 1C, JIK does not belong to any of the STE20-related branches but, together with its C. elegans homolog, it defines an independent subfamily.

Although JIK was initially isolated by virtue of its ability to bind to the bacterially expressed SH3 domain of eps8, no interaction between eps8 and JIK was observed when JIK was co-expressed in mammalian cells together with eps8, or when lysates from JIK-expressing cells were affinity-purified with bacterially expressed eps8 (data not shown). Thus, we concluded that JIK binds eps8 in vitro, but that this is likely to represent a low affinity interaction. The biological relevance of such an interaction, as well as whether JIK can physically associate in vivo to other eps8-related proteins, is under current investigation.

Analysis of the cDNA and Expression of JIK mRNA—To examine the tissue distribution of JIK, we performed Northern blot analysis to establish the level of JIK mRNA expression in several human tissues. As shown in Fig. 2, a major distinct transcript of 4.4 kilobase pairs was detected in most tissues. The levels of JIK mRNA are similar in the tissues analyzed, with the exception of low level expression in the skeletal mus-
undergo autophosphorylation and dramatically phosphorylate MBP, as compared with the wild type JIK.

To further ascertain the nature of the phosphorylated residues in JIK, autophosphorylated JIK was subjected to phosphoamino acid analysis. As shown in Fig. 4B, autophosphorylated JIK contained both phosphoserine and phosphothreonine, but not phosphotyrosine. Together, these results suggested that JIK is a serine/threonine kinase.

JIK Is Negatively Regulated by EGF—Although JIK was

Fig. 2. Expression of JIK mRNA in human tissues. A multiple tissue Northern blot was hybridized with JIK cDNA probe (upper panel), stripped, and re-hybridized with a human glyceraldehyde 3’-phosphate dehydrogenase probe (lower panel). Each lane represents 2 μg of poly(A)+ RNA isolated from the indicated tissues. Molecular size markers are shown on the left in kilobase pairs.

Fig. 3. Immunoprecipitation and immunoblot analysis of JIK. 5 mg of cell lysates from asynchronously growing NIH-3T3 and C33A cells were immunoprecipitated with polyclonal anti-JIK (lanes 3 and 4) and with preimmune rabbit serum (lanes 1 and 2). 5 mg of cell lysates from C33A cells transfected with vector alone (lane 5) or with HA-tagged JIK (lane 6) were immunoprecipitated with anti-HA antibody. The immunoprecipitants were separated by SDS-PAGE (10%) and, after transferring onto Immobilon P membrane, were immunoblotted with anti-JIK antibody.

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JIK Is Negatively Regulated by EGF—Although JIK was
enzymatically active when expressed in COS7 cells, we set out to investigate whether a variety of exogenous stimuli, including stress inducing agents such as UV light, NaCl, H2O2, TNF-α, anisomycin or Lα-Lysophosphatidic acid, were modulating the catalytic activity of JIK. In this initial search, we found that none of these stimuli were able to cause the elevation of the kinase activity of JIK (data not shown). We next examined the response of JIK upon treatment with human epidermal growth factor (EGF). AU5-tagged JIK was transiently expressed in COS7 cells and its kinase activity was analyzed upon stimulation with EGF at different times. As shown in Fig. 5, JIK enzymatic activity was still considerably high in response to EGF treatment for 5 min, when compared with the level of activation detected in unstimulated cells. However, JIK kinase activity and its autophosphorylation level were found to be potently reduced upon 15 min of EGF stimulation. Interestingly, the blockade of JIK kinase activity elicited by EGF was still detectable upon 20, 25 and 30 min of stimulation (not shown). Moreover, as shown in the bottom panel of Fig. 5, levels of expression of JIK were not affected by these treatments. Thus, taken together, these findings indicate that EGF might negatively regulate JIK activity.

JIK Does Not Modulate ERK2, ERK6, p38, SAPK4, and ERK5 Activity—To investigate whether JIK plays a role in regulating the activity of signaling kinases, in vitro kinase assays were performed using lysates from cells coexpressing AU5-tagged JIK cDNA together with HA-epitope tagged cDNAs encoding either ERK2, ERK6, p38, SAPK4 or ERK5. The activity of the immunoprecipitated MAPKs was determined by measuring the phosphorylation of MBP for ERK2, ERK6, p38 and ERK5, and GST-ATF2(96) for SAPK4. As shown in Fig. 6a, coexpression of wild type JIK with ERK2 did not cause enhancement of MBP phosphorylation, as compared with the negative control. However, since it has been demonstrated that ERK2 catalytic activity is potently enhanced in response to EGF (85) and since we showed that JIK kinase activity is dramatically blocked by treatment with EGF, we set out to investigate the effect of JIK on ERK2-elicited stimulation in EGF-treated cells. As shown in Fig. 6A, as compared with the control coexpressed with the empty vector, JIK does not interfere with the activation of ERK2 elicited by EGF. Furthermore, we confirmed that JIK does not activate nor have a significant
negative affect on the enzymatic activity of ERK2, ERK6, p38, SAPK4, and ERK5.

**JIK Inhibits JNK/SAPK in an EGF-, Anisomycin-dependent, but UV-independent Manner by Acting on a Step Upstream from the Small GTPases—**

Many members of the STE20-like kinase family have been shown to play a role in the activation of the SAPK/JNK cascade. Therefore, to examine the ability of JIK to affect the SAPK/JNK pathway, we coexpressed AU5-tagged JIK with HA-tagged JNK in COS7 cells. The activity of JNK was determined by immune complex kinase assay using GST-ATF2(96) as a substrate. Unlike other STE20-like kinases, JIK was unable to activate JNK/SAPK, but, interestingly, JIK was found to reduce the basal activity of JNK/SAPK (4.2-fold). The decrease in SAPK/JNK activity was not due to variation of JNK expression since similar levels were detected in all samples analyzed (Fig. 7).

**Fig. 6. JIK does not activate neither inhibits ERK2, ERK6, p38, SAPK4, and ERK5.** A, subconfluent COS7 cells were cotransfected with pCDNA3-HA-ERK2 together with empty vector (control) (lanes 1 and 4), pCEFL-AU5-JIK (lanes 2 and 5), or pCEFL-AU5-JIK(A181 F183) (1 μg each), starved overnight and treated with EGF (100 ng/ml, 10 min), as indicated. Lysates were then subjected to in vitro kinase assay, using MBP as substrate. Detection of HA-ERK2, AU5-JIK, and AU5-JIK(A181 F183) in the lysates was determined, respectively, after immunoprecipitation with anti-HA and anti-AU5 and immunoblotting with anti-ERK2 and anti-JIK antisera. B–E, Subconfluent COS7 cells were cotransfected with pCEFL-HA-ERK6, pCEFL-HA-p38, pCEFL-HA-SAPK4, or pCEFL-HA-ERK5 (1 μg/plate) together with empty vector (control) (lanes 1 and 4) or pCEFL-AU5-JIK (lanes 3 and 4) (1 μg each). Cells were starved overnight and treated, where indicated, respectively with NaCl (300 mM, 20 min) for the ERK6 assay (B), anisomycin (10 μg/ml, 20 min) for p38 and SAPK4 assays (C and D, respectively) and H₂O₂ (200 μM, 20 min) for ERK5 assay. Lysates were then subjected to in vitro kinase assay, using MBP (B, C, E) or GST-ATF2(96) (D) as exogenous substrates. Detection of HA-tagged form of ERK6, p38, SAPK4, ERK5, and AU5-JIK in the lysates was determined respectively after immunoprecipitation with anti-HA and anti-AU5 and immunoblotting with anti-HA (B, D), anti-p38 (C), or anti-ERK5 (E) and anti-JIK antisera.
tion. Upon coexpression of HA-JNK with AU5-JIK in COS7 cells, the cells were subsequently stimulated with 100 ng/ml of EGF for 15 min. It has already been shown that JNK/SAPK is fully activated upon 15 min of EGF treatment in COS7 cells

(55) and the same time of stimulation is sufficient to cause a dramatic reduction of JIK kinase activity (Fig. 5). Stimulation with EGF for 15 min led to a 2.3-fold increase of JNK activity, when compared with unstimulated cells (Fig. 7A). In contrast, when coexpressed with JIK, as expected, JNK was only marginally activated by EGF. To ascertain that the JIK kinase activity was indeed responsible for the JNK/SAPK inhibition, we coexpressed HA-tagged JNK together with JIK catalytically inactive mutant [AU5-JIK(A181 F183)]. In these cells, the basal level of JNK activity was not inhibited, as compared with the level of activation detected in cells coexpressing wild type JIK and JNK. Furthermore, in the presence of the mutated form of JIK, the EGF-elicited activity of JNK was only slightly lower than in cells transfected with vector alone, most likely due to the fact that JIK(A181 F183) still retains some kinase activity (Fig. 4A). Immunoblot analysis detected the same level of expression of JNK, JIK and JIK(A181 F183) in the samples analyzed.

Since it has also been reported that both anisomycin and UV light are potent activators of JNK/SAPK pathway (8–10, 55), we next explored the effect of JIK on JNK/SAPK cascade, upon stimulation with these two stress-inducing agents. As shown in Fig. 7B, both anisomycin and UV light caused a potent activation of JNK/SAPK cascade, when compared with the unstimulated cells, as determined by GST-ATF2(96) phosphorylation. However, the overexpression of JIK did not significantly affect the activation of JNK/SAPK elicited by anisomycin or UV light. As shown in the bottom panels, immunoblot analysis of the immunoprecipitants indicated that the treatment with these stimuli did not alter the expression of JIK, JIK (A181F183), or JNK.

These observations suggest that JIK might act in a JNK/SAPK-inhibitory pathway, by reducing the basal state of JNK/SAPK activation and partially preventing the response of JNK/SAPK to EGF. In contrast, our data demonstrate that JIK does not modulate the activation of molecules mediating the induction of JNK/SAPK signaling pathway by chemical and physical stresses.

It has been recently demonstrated that the constitutively active forms of the small GTP-binding proteins Rac1 and Cdc42 (Rac1 QL and Cdc42 QL, respectively) led to a potent stimulation of the JNK/SAPK signal transduction pathway (55). Moreover, it has been also shown that the overexpression of the MAPK kinase kinase 1 (MEKK1) as well as the mixed lineage kinase 3 (MLK3) are sufficient to enhance JNK/SAPK cascade (25, 86). Thus, to further investigate the mechanism whereby JIK inhibits JNK activation, we sought to determine whether the activation of JNK/SAPK pathway elicited either by Rac1 QL, Cdc42 QL, MLK3, or MEKK1 was affected by the overexpression of wild type form of JIK. As shown in Fig. 8, JIK was not able to inhibit the activation of JNK/SAPK signal transduction pathway upon Rac1 QL (Fig. 8A), Cdc42 QL (Fig. 8B), MLK3 (Fig. 8C), or MEKK1 (Fig. 8D) induction. The expression levels of the immunoprecipitated JNK/SAPK and JIK were examined by immunoblot analysis and were confirmed to be present at comparable amounts in the samples analyzed. These findings indicate that JIK might exert its inhibitory function by acting on a step upstream from the small GTPases Rac1 and/or Cdc42.

**DISCUSSION**

In this study we describe the cloning of a novel human serine/threonine kinase, designated JIK (for JNK/SAPK-inhibitory kinase), which was found to be ubiquitously expressed in human tissues. Based on its structure, this novel kinase was determined to belong to the STE20-related family of serine/threonine kinases and, in particular, to the GCK-like subfamily...
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of STE20 homologs. In its overall structure, JIK is highly similar to a putative C. elegans serine/threonine kinase, termed SULU, which has not been yet characterized. Interestingly, the obtained dendrogram, based on the computer-assisted alignment of the catalytic domain of each of the STE20-related kinases, showed that JIK, together with its C. elegans homolog, did not belong to any of the known STE20-related branches, but instead determined an independent subfamily.

When overexpressed in C33A and COS7 cells, JIK was shown to encode a protein of 110 kDa with autophosphorylating properties. Phosphoamino acid analysis of phospholabeled proteins indicated that JIK was indeed a serine/threonine kinase and, when expressed in COS7 cells, JIK was found to be enzymatically active. However, systematic evaluation of physical conditions, ligands, or substances activating other members of the STE20 family of kinases revealed that no exogenous stimuli could result in elevated JIK kinase activity. In contrast, we found that EGF receptor stimulation causes a dramatic decrease in JIK activity. However, EGF did not cause the tyrosine phosphorylation of JIK nor did it promote the association of JIK with EGF receptors or other phosphotyrosine-containing proteins (data not shown). Thus, how activation of EGF receptor modulates JIK activity is currently unknown and under investigation. We can nevertheless conclude that JIK may represent the first STE20-like kinase that can be negatively regulated after activation of a cell surface receptor, and most likely, the first STE20-related kinase that responds to growth factors, rather than to stress pathways.

Germinal center kinase (GCK) (64) as well as germinal center kinase-like kinase (GLK) (61), are reported to activate the JNK/SAPK pathway through activation respectively of SEK1 and MEKK1, but unable to activate p38 or ERKs. Another member of STE20-related kinase, KHS, is also able to induce the activation of the JNK/SAPK kinase cascade, but not p38 or ERK2 kinases. Mouse and human HKP1 have been shown to induce JNK/SAPK cascade (62, 63), via the SH3-containing mixed lineage kinase MLK-3 and MAPK/ERK kinase 1 (MEKK1), which both in turn activate SEK1 (also called MKK4 or JNKK) (21, 87). Interestingly, these four kinases all belong to the same evolutionary branch and function as upstream activators of JNK/SAPK pathway, suggesting that they may have developed during evolution to transmit signals to JNK in response to distinct stimuli. Another STE20-related kinase, NIK (Nck-interacting kinase), was recently shown to activate JNK/SAPK but not ERK2 (58). Taken together, these findings indicate that many STE20-related kinases are likely to specifically function in the activation of JNK/SAPK pathway. However, the function of many of the other members of this family of kinases, such as SOK1, LOK1, SLK, MST1, MST2, and MST3, remains yet to be elucidated. In this regard, the highly divergent structure of these proteins outside their kinase domain suggests that they probably respond to, and are regulated by, very different cellular elements. Unlike other members of the GCK-like family that activate JNK/SAPK pathway, JIK was found to reduce the basal level of JNK/SAPK activity more than 4-fold, whereas kinase-inactive JIK had no demonstrable effect. In addition, the stimulation of JNK in response to EGF treatment was partially inhibited by the coexpression of JIK. Moreover, we found that JIK did not significantly reduce anisomycin- or UV-elicited JNK/SAPK activation, thus suggesting that JIK may represent a regulatory member of a novel inhibitory pathway, controlling the constitutive or the EGF-mediated activation of JNK/SAPK cascade. Interestingly, the JIK kinase activity was required for the inhibition of JNK/SAPK constitutive activity. However, EGF itself has been shown to reduce JIK enzymatic activity after 15 min of ligand addition. Furthermore, upon 10 min of EGF stimulation, JIK was demonstrated to be still in its active state (data not shown) and thus was able to block JNK/SAPK basal activation. This may explain the EGF-mediated JNK/SAPK partial activation observed when wild type JIK was coexpressed.

A number of molecules have been recently shown to exert a negative effect on the JNK/SAPK signaling pathway. They include glutathione S-transferase P (GSTp), which physically associates with and inhibits JNK/SAPK (88), JNK-inhibitory protein (JIP-1); which acts as a scaffolding molecule facilitating the activation of JNK/SAPK but preventing its translocation to the nucleus (89); thioredoxin, which binds and inhibits Ask-1, a molecule acting upstream of JNK/SAPK kinase (90); and the G-protein pathway suppressor 2 (GSP2), which blocks the activity of JNK/SAPK by TNF-α, whose molecular target is still unknown (91). For JIK, co-immunoprecipitation experiments failed to demonstrate a direct association with JNK/SAPK (data not shown). Furthermore, when we examined the effect of JIK on signaling molecules acting upstream from JNK/SAPK, we found that JIK did not affect the stimulation of JNK/SAPK by the constitutively active form of Rac1 and Cdc42 (Rac1 QL and Cdc42 QL, respectively), nor the elevated JNK/SAPK activity in response to EGF (data not shown). Furthermore, when examined the effect of JIK on signaling molecules acting upstream from JNK/SAPK, we found that JIK did not affect the stimulation of JNK/SAPK by the constitutively active form of Rac1 and Cdc42 (Rac1 QL and Cdc42 QL, respectively), nor the elevated JNK/SAPK activity in response to the expression of MEKK1 or MKK4, and ERK5 function or exerted very limited inhibitory effect on p38 and ERK5. Nevertheless, in no case JIK caused any increase in the level of activation of these members of the MAPK superfamily.

The function(s) of the C-terminal regulatory domain of JIK are yet to be identified. We nevertheless speculate that the JIK regulatory domain might recruit interacting molecules, such as protein phosphatases, which may trigger the dephosphorylation and inactivation of downstream targets, thereby leading to the inhibition of JNK/SAPK signal transduction pathway. In addition, although the sequence analysis of the C terminus of JIK revealed no known protein-protein interaction motifs, the
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nature of the molecular targets for JIK in this novel JNK/SAPK pathway.

In summary, our data suggest that JIK represents the first inhibitor of the JIK/SAPK pathway.

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