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Activation of the c-Jun N-terminal kinase pathway by a novel protein kinase related to human germinal center kinase

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ABSTRACT **The c-Jun N-terminal kinase (JNK), or stress-activated protein kinase plays a crucial role in cellular responses stimulated by environmental stress and proinflammatory cytokines. However, the mechanisms that lead to the activation of the JNK pathway have not been elucidated. We have isolated a cDNA encoding a novel protein kinase that has significant sequence similarities to human germinal center kinase (GCK) and human hematopoietic progenitor kinase 1. The novel GCK-like kinase (GLK) has a nucleotide sequence that encodes an ORF of 885 amino acids with 11 kinase subdomains. Endogenous GLK could be activated by UV radiation and proinflammatory cytokine tumor necrosis factor α. When transiently expressed in 293 cells, GLK specifically activated the JNK, but not the p42/44^{MAPK}/extracellular signal-regulated kinase or p38 kinase signaling pathways. Interestingly, deletion of amino acids 353–835 in the putative C-terminal regulatory region, or mutation of Lys-35 in the putative ATP-binding domain, markedly reduced the ability of GLK to activate JNK. This result indicates that both kinase activity and the C-terminal region of GLK are required for maximal activation of JNK. Furthermore, GLK-induced JNK activation could be inhibited by a dominant-negative mutant of mitogen-activated protein kinase kinase kinase 1 (MEKK1) or mitogen-activated protein kinase kinase 4/SAPK/ERK kinase 1 (SEK1), suggesting that GLK may function upstream of MEKK1 in the JNK signaling pathway.**

The mitogen-activated protein kinase (MAPK) cascades are highly conserved in all eukaryotes and participate in various intracellular signaling pathways that control a wide spectrum of cellular responses including cell growth, differentiation, and stress responses (1–3). MAPKs are a family of serine/threonine protein kinases that include p42/44^{MAPK} [also referred to as extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2)] (4, 5), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) (6–8), p38 kinase (9, 10) and ERK5 (11). The mammalian p42/44^{MAPK} is involved in growth factor-mediated activation and differentiation of a variety of cells, and is activated by growth factors and mitogens such as epidermal growth factor, insulin, and phorbol esters (4). MAPK is activated by phosphorylation of both threonine and tyrosine residues catalyzed by its upstream protein kinase known as MAPK kinase or MEK (12). MEK itself is activated by phosphorylation on two conserved serine residues by several distinct mammalian serine/threonine kinases, including Raf (13–15), Mos (16), and MEK kinase 1 (MEKK1) (17).

JNK/SAPKs are identified as new members of the MAPK family. JNK phosphorylates several transcription factors including c-Jun (18), ATF2 (19), and ELK-1 (20) and regulates their activities. Distinct from the p42/44^{MAPK}, JNK is predominantly activated in response to stress-inducing signals such as osmotic and heat shock, UV light, protein synthesis inhibitors, and proinflammatory cytokines (6–8). JNK is activated by phosphorylation of both threonine and tyrosine residues by its upstream MAPK kinase, MKK4/SAPK/ERK kinase 1 (SEK1) (21–23). MKK4 is itself regulated by phosphorylation by several upstream MAPK kinase kinases including MEKK1, -2, -3, and -4 (17, 24–27), Tpl-2 (28), and MAPKKK5 (29). The exact mechanism that couples the signals from extracellular stimuli to the JNK signaling cascade to achieve specific responses to different stimuli remains to be elucidated.

In the p42/44^{MAPK} pathway, the Ras superfamily of small GTPases transmits signals from extracellular stimuli and regulates the p42/44^{MAPK} pathway (30). In the GTP-bound state, Ras binds directly to Raf (15) and recruits Raf to the plasma membrane (31). Whereas Ras controls the activation of p42/44^{MAPK}, recent studies reported that two Ras-like small GTP-binding proteins, Rac1 and Cdc42, can stimulate JNK in several cell lines, presumably by activating other kinases (32, 33). However, the downstream targets of Cdc42/Rac that are responsible for JNK activation have not been clearly identified.

Studies in the budding yeast suggested that the potential target for Cdc42/Rac1 might be the serine/threonine kinase, Ste20. In the *Saccharomyces cerevisiae* pheromone response, Ste20 acts at an early step in a signaling cascade that includes Ste11 (MAPKKK), Ste7 (MKK), Fus3 (MAPK), and Kss1 (MAPK) (34). Ste20 activates this pathway in response to signals from both Cdc42 and heterotrimeric G proteins associated with transmembrane pheromone receptors (34, 35). Thus, Ste20 may serve as a connection point for the heterotrimeric G proteins and Cdc42.

Several mammalian Ste20-like kinases have been identified to date, including the human p21-activated protein kinase (PAK1) (36, 37), MST1 and -2 (38, 39) or KRS (40), germinal center kinase (GCK) (41, 42), hematopoietic progenitor kinase 1 (HPK1) (43, 44), KHS (45), SOK (46), and NIK (47). This family of kinases can be classified into two subfamilies based on their structures and regulation. The kinases in the first class are closely related to yeast Ste20 in structure with a catalytic domain in the C terminus and a binding site for Rac1 and Cdc42 in the N terminus (48). The second class of kinases has a catalytic domain

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Abbreviations: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MKK or MEK, MAPK kinase; GCK, germinal center kinase; GLK, GCK-like kinase; HPK1, hematopoietic progenitor kinase 1; PAK, p21-activated protein kinase 1; HA, hemagglutinin; MBP, myelin basic protein; SAPK, stress-activated protein kinase; SEK1, SAPK/ERK kinase 1; GST, glutathione *S*-transferase; TNF-α, tumor necrosis factor α.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF00145).

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in the N terminus and does not appear to contain the Cdc42 and Rac1 binding sites. The prototype for the first class of kinases is PAK1. The second class includes MST1 and -2, GCK, HPK1, KHS, SOK, and NIK. Little is known about their activators, regulatory mechanisms or physiological roles of the second class of kinases.

We report here the isolation of a novel human protein kinase, referred to as GCK-like kinase (GLK), which belongs to the second class of the Ste20 family of kinases. Expression of GLK specifically activates the JNK/SAPK pathway in transfected 293 cells. Furthermore, dominant-negative forms of MEKK1 and MKK4/SEK1 inhibited GLK-induced JNK activation, suggesting that GLK may function upstream of MEKK1.

MATERIALS AND METHODS

cDNA Cloning and Northern Blot Analysis. Degenerate oligonucleotide primers were used in a PCR using human skeletal muscle cDNA as template as described (29). A DNA fragment having significant homology to protein kinases was identified and used to screen a human skeletal muscle library in λ phage. Replicate filters were prehybridized for 2 h at 42°C in 5× standard saline citrate (SSC), 1× Denhardt's solution containing 100 μg/ml of salmon sperm DNA, 50% formamide, and 0.1% SDS and hybridized overnight in the same solution to a 180-bp probe labeled with [α-^{32}P]dCTP. Filters were washed twice (two times for 30 min) in 0.1× SSC/0.1% SDS at 55°C. Positive clones were picked, purified, and sequenced on both strands.

A Northern blot filter containing poly(A)$^+$ RNA (2 μg/lane) from multiple tissues (CLONTECH) was probed with a ^{32}P-labeled DNA fragment generated from the 3′ end of the coding region of GLK. Hybridization was performed at 68°C in Express Hybridization Buffer (CLONTECH) followed by three washings in 0.1% SSC/0.1% SDS at 55°C.

Plasmid Construction. Full-length GLK was cloned into mammalian expression vectors PCR3.1 (Invitrogen) and pCI (Promega) by PCR using two oligonucleotide primers. The primers added a flag epitope sequence at the 5′ end. A catalytically inactive mutant of GLK was created by substituting Lys-35 with a glutamic acid (K35E) by site-directed mutagenesis using the overlapping PCR method as described (29). A C-terminal deletion mutant was generated by removing a *Bgl*II–*Bgl*II fragment in the pCI-GLK plasmid followed by religation. The MEKK1-DN plasmid had been described (43).

Generation and Affinity Purification of GLK Antibody. A synthetic peptide (CQQQNEHRGTNLSRKEKKDV) corresponding to the peptide sequence 499–518 of GLK was synthesized and coupled to keyhole limpet hemocyanin via the cystein residue at the N terminus and used to immunize rabbits and serum was collected. The same peptide was covalently linked to Affi-gel 15 (Bio-Rad) according to the manufacture's protocol. Serum was incubated with the peptide resin for 2 h. The column was then washed with 5 volumes of TBS (20 mM Tris/150 mM NaCl, pH 7.5) until absorbance reached the baseline. Antibody was eluted with three volumes of 100 mM glycine (pH 3.0) and neutralized with 1 M Tris (pH 8.0).

Cell Culture and Transfection. 293 cells were grown in DMEM supplemented with 10% FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin. For transfection, 2×10^6 cells were plated onto 100-mm dishes 16–20 h before transfection. DNA (5.0 μg) was transfected using LipofectAMINE (Life Technologies, Grand Island, NY). Transfected cells were incubated for 5 h in serum-free DMEM, further incubated in DMEM with 10% fetal calf serum, and harvested 48 h after transfection.

JNK/ERK/p38 Kinase Activity Assays. Cells cotransfected with GLK and hemagglutinin (HA) epitope-tagged JNK1, Erk2, or p38 kinase plasmid were lysed and recombinant protein was immunoprecipitated using mAb to the HA epitope and protein A-Sepharose CL-4B beads. Beads were washed three times with lysis buffer, once with kinase buffer (25 mM Hepes, pH 7.4, 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 25 mM DTT, and 0.1 mM Na_3VO_4). The beads were then incubated with human glutathione *S*-transferase (GST) c-Jun (1–169) (Upstate Biotechnology, Lake Placid, NY), PHAS-I (Stratagene) or ATF2 peptide (1–109) and 10 μCi of [γ-^{32}P]ATP (1 Ci = 37 GBq) at 30°C for 30 min. Reaction mixtures were then resuspended in 2× sample buffer (125 mM Tris/6% SDS/20% glycerol) and boiled at 100°C for 3 min. Phosphorylated proteins were analyzed by SDS/PAGE.

***In Vitro* Protein Kinase Assays.** Cell lysates were immunoprecipitated with M2 mAb and protein A-Sepharose CL-4B beads as described above. The immunoprecipitated kinases were resuspended in 40 μl of reaction buffer (20 mM Mops, pH 7.2/25 mM β-glycerol phosphate/5 mM EGTA/1 mM Na_3VO_4/1 mM DTT). Thereafter, 10 μl of reaction buffer containing 5 μg of myelin basic protein (MBP), or polyhistidine MEKK1 (1–301) fusion proteins (Santa Cruz Biotechnology), 75 mM $MgCl_2$, and 500 mM ATP along with 10 μCi [γ-^{32}P]ATP were added and incubated at 30°C for 30 min.

Time Course of GLK Activity with UV Radiation or Tumor Necrosis Factor α (TNF-α) Stimulation. HeLa cells were seeded at a concentration of 2.3×10^5 cells/ml onto 6-well plates. Forty hours after seeding, cells were stimulated with either TNF-α at 15 ng/ml or 80 J/M^2 of UV radiation, collected at the indicated time points, and lysed. Cell lysates (50 μg) were precleared with protein A beads and nonspecific antibodies overnight. GLK was then immunoprecipitated with 1.4 μg of anti-GLK antibody for 12 h. The immunoprecipitates were washed two times with cell lysis buffer, LiCl buffer, and kinase buffer. Kinase reactions were performed for 40 min in kinase buffer containing 10 μCi [γ-^{32}P]ATP, 15 μM cold ATP, and 5 μg of MBP.

RESULTS

Molecular Cloning of GLK. Degenerate oligonucleotide primers were designed based on conserved amino acid sequences in the catalytic domains of the serine/threonine protein kinases and were used to amplify cDNA using human skeletal muscle cDNA as template. A PCR fragment containing novel sequences that has homology to the Ste20 family of protein kinases was amplified. This cDNA fragment was subsequently used to screen a human skeletal muscle cDNA library and several cDNA clones were isolated; the clone containing the longest insert (4.2 kb) was sequenced. The nucleotide sequence of the cDNA predicts an ORF of 885 amino acids with a predicted molecular mass of 100 kDa (Fig. 1*A*). The predicted initiator methionine is preceded by a purine at the −3 position and a purine at the +4 position, making it optimal for translational initiation (data not shown) (49). There are in-frame stop codons at the 5′ end (data not shown). The deduced amino acid sequence predicts a kinase catalytic domain at the N terminus and contains 11 kinase subdomains. GenBank and EMBL database searches identified human GCK and HPK1 as the molecules most closely related to GLK. Human GLK shares 57% amino acid identity with GCK and 49% amino acid identity with HPK1. Within the kinase domain, GLK displays 72%, 66%, 42%, and 41% amino acid identities to the catalytic domains of human GCK, HPK1, PAK1, and *S. cerevisiae* Ste20 (35, 50), respectively. The amino acid sequence alignment of the catalytic domain of GLK with that of related kinases is shown in Fig. 1*B*. The C-terminal regulatory domain of GLK contains three proline-rich sequence motifs that are putative SH3 domain binding sites (Fig. 1) (51, 52). In addition, two PEST (proline, glutamic acid, serine, and threonine) sequences were found in the middle of GLK. These may contribute to rapid protein degradation (Fig. 1) (53).

Tissue Distribution of GLK mRNA. The expression of GLK was examined in a variety of human tissues by Northern blot analysis using a probe from the noncatalytic C-terminal region of GLK. The GLK probe hybridized to a single transcript of ≈4.2 kb in all tissues examined (Fig. 2).

Characterization of GLK Protein. Full-length GLK cDNA was cloned into a mammalian expression vector with a flag epitope

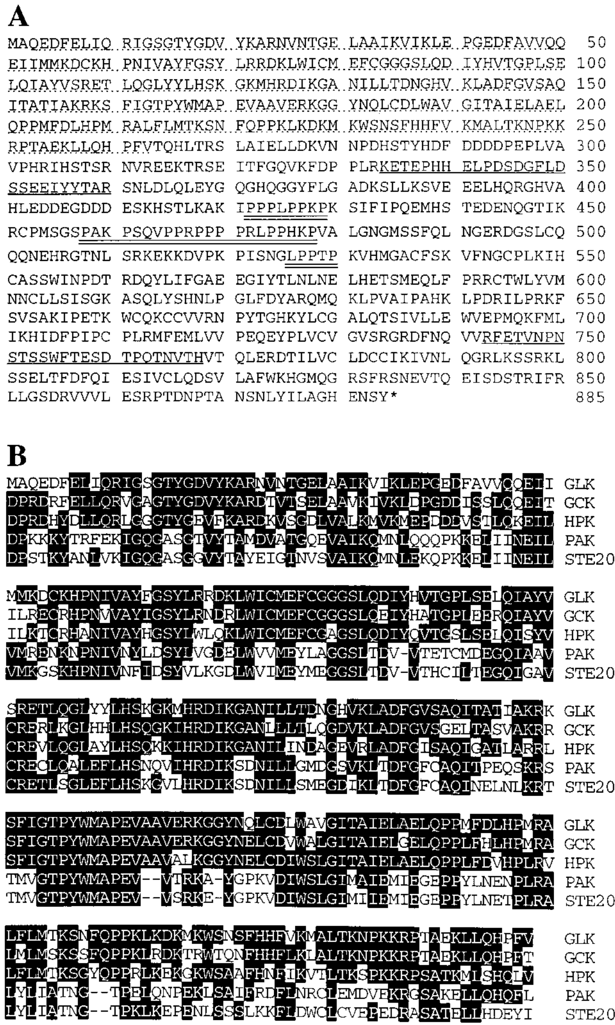

FIG. 1. (*A*) Deduced amino acid sequences of GLK. The deduced amino acid sequence of GLK is shown. The putative catalytic domain is underlined (..). Three proline-rich regions are doubly underlined (═). Two putative PEST (proline, glutamic acid, serine, and threonine) sequences are singly underlined (—). (*B*) Amino acid sequence alignment and comparison of GLK with the related protein kinases. The putative catalytic domain of GLK was aligned and compared with the catalytic domains of human GCK, HPK1, PAK1, and *S. cerevisiae* Ste20 by the DNASTAR program.

sequence added at the 5′ end and transfected into 293 cells. Transfected cell lysates were then immunoblotted with a mAb to the flag epitope. The flag mAb specifically recognized a 100-kDa protein in cells transfected with GLK, but not in cells transfected with vector alone (Fig. 3*A*, lanes 1 and 2). The size of the protein corresponds to the predicted molecular mass of GLK.

To determine whether GLK has protein kinase activity, transfected cell lysates were subjected to immunoprecipitation with a flag mAb and used in an immune complex kinase assay. Although the immunoprecipitates from cells transfected with vector alone phosphorylated MBP, the kinase activity was markedly increased in immunoprecipitates from cells transfected with GLK (Fig. 3*B*, lanes 1 and 2). In addition to the phosphorylated form of MBP, a phosphoprotein of 100 kDa was observed only in the cells transfected with GLK (Fig. 3*B*, lane 2), suggesting that GLK can autophosphorylate. To eliminate the possibility that an associated kinase might be coprecipitating with GLK which may account for the kinase activity, we generated a kinase inactive form of GLK by replacing Lys-35 in the ATP binding domain with a glutamic acid (K35E) and tested it in the same immune complex kinase assay. The level of phosphorylation of MBP by the K35E mutant

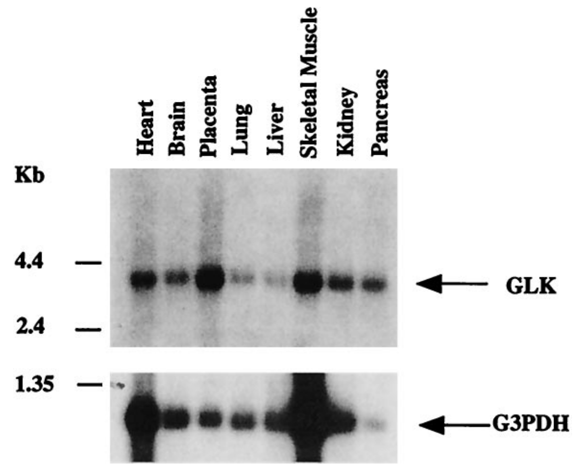

FIG. 2. Expression pattern of human GLK mRNA. A filter containing poly(A)$^+$ RNA from the indicated tissues was hybridized with a radioactive GLK probe as described. The hybridization with a glyceraldehyde-3-phosphate dehydrogenase (G3PDH) probe was also shown.

was similar to that of the control (Fig. 3*B*, lane 3). In addition, the K35E mutant did not autophosphorylate (Fig. 3*B*, lane 3). These data indicate that GLK is a functional protein kinase. Unlike GCK, GLK did not phosphorylate casein and histone II (data not shown).

Activation of JNK, But Not ERK2 or p38 Kinase in Cells Transfected with GLK. To examine whether GLK can activate the mammalian JNK cascade, we cotransfected 293 cells with mammalian expression plasmids encoding human GLK and an HA epitope-tagged JNK. Recombinant JNK was immunoprecipitated from cell lysates and used in a protein kinase assay with GST c-Jun protein as substrate. Transfection of cells with GLK resulted in strong activation of JNK (Fig. 4*A*, lane 2), while cells transfected with vector alone had no effect (Fig. 4*A*, lane 1). The level of activation was comparable to that of cells stimulated with 10 ng/ml of anisomycin (Fig. 4*A*, lane 3). Western blot analysis showed that JNK was expressed at comparable levels (data not shown). To rule out the possibility that GLK might coprecipitate with JNK and phosphorylate GST c-Jun, GLK was immunoprecipitated from cell lysates and used in a protein kinase assay with GST c-Jun as substrate. Although we observed the autophosphorylated GLK, we did not detect the phosphorylation of GST c-Jun by GLK (Fig. 4*B*, lane 2).

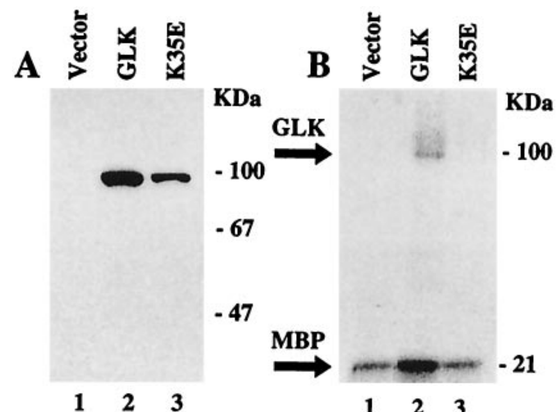

FIG. 3. Characterization of human GLK. (*A*) 293 cells were transfected with vector alone (lane 1), an expression vector expressing GLK tagged with an N-terminal flag epitope (lane 2), or a kinase inactive mutant (K35E) (lane 3). Cell lysates were harvested and immunoblotted with mAb M2. (*B*) Lysates from cells transfected with vector alone (lane 1), wild-type GLK (lane 2), or K35E mutant (lane 3) were prepared. GLK was immunoprecipitated with mAb M2, then used in a kinase assay with MBP as substrate.

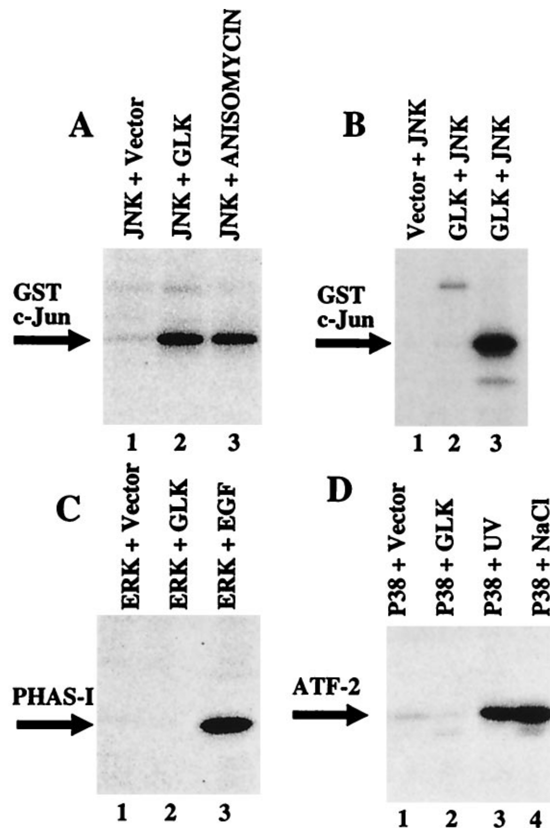

FIG. 4. Activation of JNK but not ERK or p38 kinase by GLK in transfected 293 cells. (*A*) 293 cells were cotransfected with either vector alone (lane 1) or GLK (lane 2) and HA epitope-tagged JNK1 plasmid. Alternatively, cells were transfected with JNK1 plasmid and treated with 10 ng/ml anisomycin (lane 3). JNK was immunoprecipitated and kinase assays were performed using GST c-Jun as substrate. (*B*) 293 cells were cotransfected with either vector alone (lane 1) or GLK (lanes 2 and 3) and HA epitope-tagged JNK1 plasmid. GLK (lane 2) or JNK (lane 3) was immunoprecipitated and used in an *in vitro* kinase assay using GST c-Jun as substrate. (*C*) 293 cells were cotransfected with either vector alone (lane 1) or GLK (lane 2) and HA epitope-tagged ERK2 plasmid. Alternatively, cells were transfected with ERK2 and stimulated with epidermal growth factor (EGF) (30 ng/ml) for 10 min (lane 3). Kinase assays were performed using PHAS-I as substrate. (*D*) 293 cells were cotransfected with either vector alone (lane 1) or GLK (lane 2) and HA epitope-tagged p38 kinase plasmid. Alternatively, cells were transfected with p38 plasmid and treated with UV light (lane 3) or 0.5 M NaCl for 30 min (lane 4). Kinase assays were performed using ATF2 peptide (1–109) as substrate.

To determine whether GLK could also activate ERK or p38 kinase, 293 cells were transiently transfected with GLK along with either HA epitope-tagged ERK2 or p38 kinase. ERK2 or p38 was then immunoprecipitated and their activities were determined by phosphorylation of either PHAS-I protein or ATF2 peptides (1–109). Addition of epidermal growth factor to 293 cells strongly activated ERK2 (Fig. 4*C*, lane 3). However, no increase in ERK2 activity was observed when GLK was overexpressed in 293 cells (Fig. 4*C*, lanes 1 and 2). Similarly, treatment of cells with UV light or osmotic shock strongly activated p38 kinase (Fig. 4*D*, lanes 3 and 4). However, no increase in p38 kinase activity was observed when GLK was overexpressed in 293 cells (Fig. 4*D*, lanes 1 and 2). Western blot analysis confirmed that GLK, ERK2, and p38 kinase were expressed in the transfected 293 cells (data not shown). Similar results were observed in COS-7 cells (data not shown). These data suggest that GLK may not play a role in the classical MAPK/ERK and p38 kinase pathways, and that the activation of the JNK pathway by GLK is specific.

Both Kinase Activity of GLK and Amino Acids 353–835 in the C Terminus Are Required for the Maximal Activation of JNK. To determine whether the kinase activity of GLK is required for JNK activation, we cotransfected 293 cells with either the wild-type GLK or the K35E mutant. Whereas transfection of cells with wild-type GLK strongly activated JNK activity (Fig. 5*A*, lane 2), transfection with the K35E mutant of GLK, in which Lys-35 in the ATP binding domain was mutated to a glutamic acid, did not result in JNK activation (Fig. 5*A*, lane 3). This result indicates that the kinase activity of GLK is required for the activation of JNK.

Structurally, GLK consists of a putative catalytic domain (amino acids 4–263) and a long putative regulatory region in the C terminus. To determine whether the C-terminal region plays a role in the GLK-induced JNK activation, we generated a deletion mutant of GLK in which amino acids 353–835 in the putative C-terminal regulatory domain was deleted. Transfection of cells with the GLK deletion mutant resulted in greatly reduced JNK activity as compared with the wild-type GLK (Fig. 5*A*, lane 4). Western blot results confirmed that both mutants were expressed to comparable levels as the wild type (Fig. 5*B*, lanes 2–4). In addition, *in vitro* kinase activity assays found that the C-terminal deletion mutant can autophosphorylate as well as phosphorylate MBP, indicating the kinase activity of the deletion mutant was not impaired (Fig. 5*C*, lane 2). The experiment was repeated five times with reproducible results. In each case, the activation of JNK by the GLK deletion mutant in transfected cells was reduced by 10 to 20-fold as compared with wild-type GLK. Taken together, these data indicate that both kinase activity and the C terminus of GLK are required for the maximal induction of JNK activity.

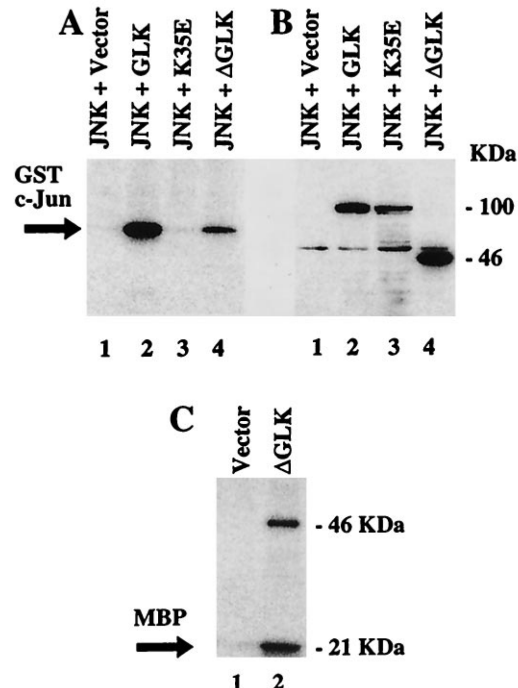

FIG. 5. Effect of kinase activity and amino acids 353–835 in the C-terminal region of GLK on JNK activation. (*A*) 293 cells were cotransfected with vector alone (lane 1), wild-type GLK (lane 2), K35E mutant (lane 3), or a GLK C-terminal deletion mutant (ΔGLK) (lane 4) plus JNK. JNK activity was assayed using GST c-Jun as substrate. (*B*) Lysates containing equal amounts of total protein were run on 10% SDS/PAGE and transferred to a nitrocellulose membrane. The blot was probed with a mAb against the flag epitope followed by rabbit anti-mouse IgG. The blot was developed using the ECL system. (*C*) Kinase activity of GLK C-terminal deletion mutant. Lysates from cells transfected with vector alone (lane 1) or with GLK C-terminal deletion mutant (ΔGLK) (lane 2) were prepared and recombinant protein was immunoprecipitated with mAb M2 followed by kinase assay with MBP as substrate.

Inhibition of GLK-Induced JNK Activation by Dominant Negative Mutants of MKK4/SEK1 and MEKK1. Because MKK4/SEK is an upstream activator of JNK which phosphorylates and activates JNK, we sought to determine whether GLK activates JNK through MKK4/SEK1. To this end, 293 cells were cotransfected with GLK and a dominant-negative mutant of MKK4/SEK1 (SEK1-AL) (21) to determine whether the dominant-negative mutant of MKK4 could inhibit the GLK induced JNK activation. Expression of a dominant negative form of MKK4 strongly inhibited the JNK activities induced by GLK (Fig. 6*A*, lane 3). Expression of wild-type MKK4 did not further increase the GLK induced JNK activation (Fig. 6*A*, lane 4), implying that the level of endogenous MKK4 was in excess in mediating JNK activation by GLK in 293 cells.

It is known that MEKK1 phosphorylates and activates MKK4/SEK1 and is a physiological activator of MKK4 *in vivo* (24, 25). We therefore sought to determine whether a dominant-negative form of MEKK1 could inhibit GLK-induced JNK activation. 293 cells were cotransfected with GLK and JNK plus a dominant-negative mutant of MEKK1 or wild-type MEKK1. JNK activities were determined using an immune complex kinase assay. Expression of a dominant-negative form of MEKK1 strongly inhibited the JNK activities induced by GLK (Fig. 6*B*, lane 4), whereas expression of wild-type MEKK1 further increased the GLK-induced JNK activation (Fig. 6*B*, lane 3). The synergistic effect between GLK and MEKK1 in activating JNK implies that the amount of endogenous MEKK1 in these cells might be limiting.

To determine whether GLK could directly phosphorylate MEKK1 *in vitro*, immunoprecipitated GLK or GLK K35E mutant was used in an *in vitro* kinase assay using MEKK1 (amino acids 1–301) as substrate. Wild-type GLK phosphorylated MEKK1 peptide, whereas the K35E mutant did not (Fig. 6*C*). Similar results were obtained in three separate experiments. Since full-length MEKK1 expressed in *Escherichia coli* or mammalian cells was constitutively active (43), it is not feasible to detect the activation of MEKK1 by GLK. Taken together, these results suggests that GLK may function upstream of MEKK1 in the JNK signaling cascade.

Activation of Endogenous GLK. A polyclonal antibody to GLK was generated and purified. This antibody specifically recognized a 100-kDa protein in cells transfected with GLK (Fig. 7*A*). The same antibody also detected a 100-kDa protein from HeLa, 293, and Ramos B cells (Fig. 7*A*).

To study the activation of endogenous GLK, HeLa cells were stimulated with UV radiation or TNF-α and cells lysates were harvested and used in an *in vitro* immune complex kinase assay. Stimulation of HeLa cells with UV radiation for 2 min

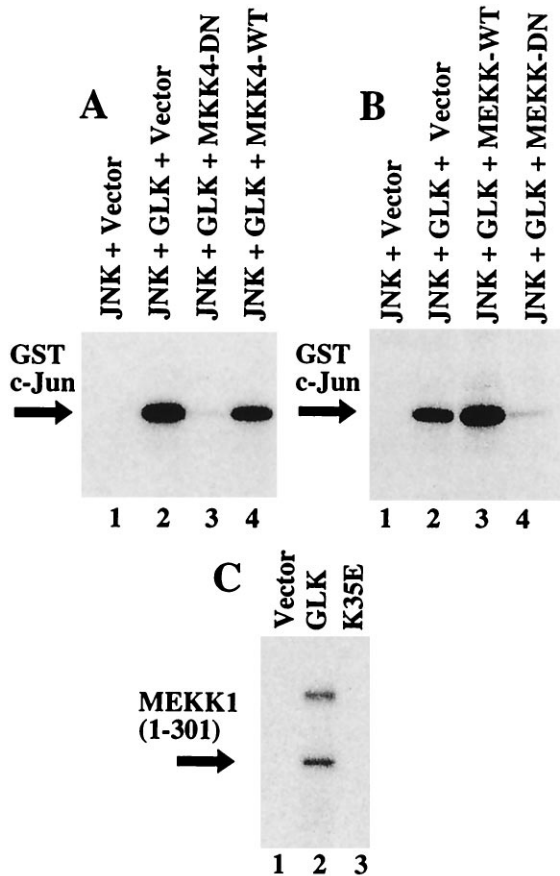

FIG. 6. Effect of dominant-negative mutants of MKK4/SEK1 and MEKK1 on GLK-induced JNK activation. (*A*) 293 cells were cotransfected with vector alone (lane 1), GLK and vector (lane 2), GLK and either wild-type MKK4 (lane 4), or a dominant-negative mutant of MKK4 (lane 3) plus HA epitope-tagged JNK. JNK activities were determined as described. (*B*) Effect of a dominant-negative mutant of MEKK1 on GLK induced JNK activation. 293 cells were transfected with vector alone (lane 1), GLK and vector (lane 2), GLK and either wild-type MEKK1 (lane 3), or a dominant-negative mutant of MEKK1 (MEKK-DN) (lane 4) plus HA epitope-tagged JNK. JNK activities were determined as described. The data are representative of three experiments performed. (*C*) Lysates from 293 cells transfected with vector alone (lane 1), wild-type GLK (lane 2), or K35E mutant (lane 3) were prepared. GLK was immunoprecipitated with mAb M2, then used in a kinase assay with a polyhistidine MEKK1 fusion protein as substrate.

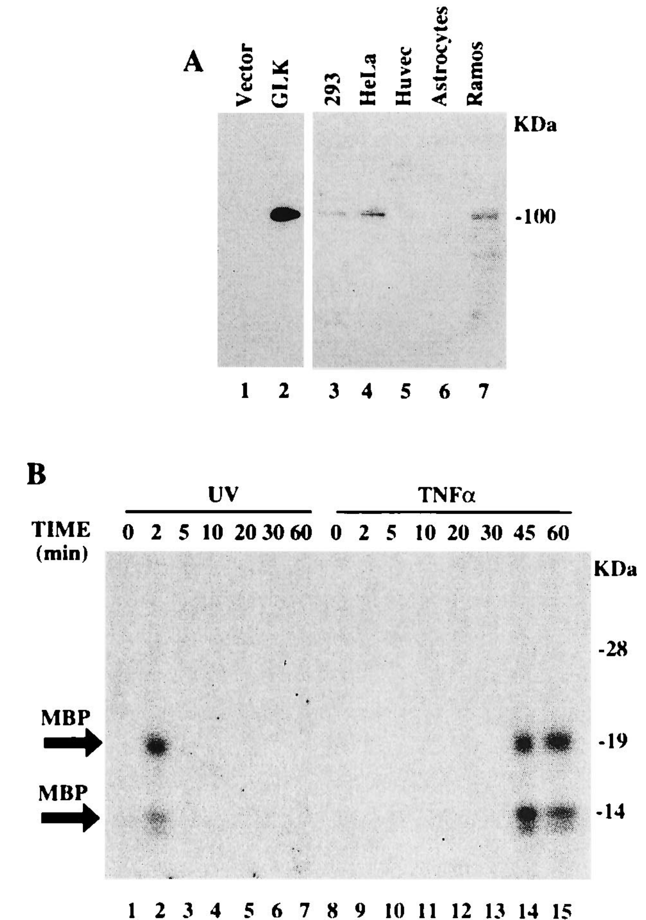

FIG. 7. Activation of endogenous GLK in HeLa cells. (*A*) Lysates from 293 cells transfected with either vector alone (lane 1) or GLK plasmid (lane 2) or from different cell lines (lanes 3–7) were immunoblotted with a polyclonal antibody to GLK. (*B*) HeLa cells were stimulated with UV radiation or TNF-α for the time indicated. Lysates containing equal amounts of total protein were immunoprecipitated with the polyclonal GLK antibody followed by a kinase assay with MBP as substrate.

or TNF-α (15 ng/ml) for 45 or 60 min markedly stimulated the kinase activity of GLK (Fig. 7*B*).

DISCUSSION

In this report, we describe the cloning and characterization of a novel member of the Ste20 family of protein kinases, which we term GLK. GLK has significant homology on the amino acid level to GCK (57% identity) and HPK1 (49% identity), which are members of the mammalian Ste20 family of kinases. In addition to sequence homology, the overall structure of GLK is also very similar to GCK and HPK1, which have a catalytic domain in the N terminus and a long putative regulatory region. The C terminus of GLK does not appear to contain Cdc42/Rac1 binding motifs that are found in the PAK kinase (48). Thus, GLK clearly fits into the second subfamily of Ste20-related protein kinases.

GCK was identified as a kinase preferentially expressed in the germinal center B cells (41), and HPK1 is predominantly expressed in hematopoietic cells, including early progenitor cells (43, 44). We studied the expression of GLK in 50 different human tissues by Northern blot analysis. GLK mRNA was found in all 50 tissues examined (data not shown and Fig. 2). The ubiquitous tissue distribution of GLK differs from that of GCK and HPK1.

Similarities among GLK, GCK, and HPK1 prompted us to investigate whether GLK can activate the three MAPK signaling pathways. We found that overexpression of GLK strongly activates the JNK pathway, but has no effect on either the MAPK or p38 kinase pathway, indicating that the activation of the JNK pathway was specific. The fact that the kinase domain of GLK only marginally activates JNK eliminated the possibility that overexpression of any Ste20-like kinase domain might activate JNK nonspecifically. The C-terminal region of GLK seems to be essential for maximal activation of JNK as the C-terminal truncation mutant of GLK has a greatly reduced ability to activate JNK. This result is in contrast to that obtained with human HPK1 in that the kinase domain alone in the human HPK1 is sufficient to activate the JNK pathway (43). With the human GCK kinase, it was found that overexpression of the C terminus alone could activate JNK (42). This is not the case with human GLK, as the K35E mutant, which contains the entire C-terminal fragment of GLK, failed to activate the JNK. It is possible that the C-terminal region of GLK might be important for binding to either upstream or downstream substrates, thus playing an important role in JNK activation. Interestingly, within the deleted region of GLK, there are three proline-rich domains which are putative SH3 binding sites. Whether these proline-rich regions play a role in binding other proteins is under current investigation.

The activation of GLK was investigated using a GLK specific antibody in an *in vitro* kinase assay. GLK was activated by known JNK activators, proinflammatory cytokine TNF-α, and UV light. In addition, the GLK K35E mutant could block TNF-α-induced activation of either endogenous JNK or cotransfected JNK (data not shown). These results are consistent with our transfection studies in that overexpression of GLK activated the JNK pathway, suggesting that GLK is a physiological activator of JNK in the responses to UV and proinflammatory cytokine. The identification of physiological activator of the Ste20-like kinases has been difficult (37, 46). Among this large group of kinases, GCK is shown to be activated by TNF-α (42). SOK can be only be activated by oxidant stress (46). MST1 can only be activated by high concentrations of sodium arsenite, heat-shock at 55°C, and okadaic acid (40). Given that a large number of diverse stimuli can activate the JNK pathway (6–8), it should not be surprising that several mammalian Ste20-like kinases can activate this pathway. It is possible that GLK, GCK, and HPK1 activate the JNK pathway in response to different upstream signals. Further studies are needed to compare the upstream activators of these different kinases and elucidate how the specificity is imparted.

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