Hematopoietic progenitor kinase 1 (HPK1) is a member of the mitogen-activated protein kinase kinase kinase (MAP4K) family and an upstream activator of the c-Jun N-terminal kinase (JNK) signaling cascade. HPK1 interacts, through its proline-rich domains, with growth factor receptor-bound 2 (Grb2), CT10-regulated kinase (Crk), and Crk-like (Crkl) adaptor proteins. We identified a novel HPK1-interacting protein of 55 kDa (HIP-55), similar to the mouse SH3P7 protein, containing an N-terminal actin-binding domain and a C-terminal Src homology 3 domain. We found that HPK1 bound to HIP-55 both in vitro and in vivo. When co-transfected, HIP-55 increased HPK1’s kinase activity as well as JNK1’s kinase activity. A dominant-negative HPK1 mutant blocked activation of JNK1 by HIP-55 showing that HIP-55 increased HPK1’s kinase activity as well as JNK1’s kinase activity. A dominant-negative HPK1 mutant blocked activation of JNK1 by HIP-55 showing that HIP-55 activates the JNK1 signaling pathway via HPK1. Our results identify a novel protein, HIP-55, that binds to HPK1 and regulates the JNK1 signaling cascade.

Mitogen-activated protein kinases (MAPKs) play essential roles in relaying extracellular signals from the plasma membrane to the nucleus of a cell. These signals control the expression of specific genes, which direct the cell to proliferate, differentiate, or respond to stress signals. The subgroups of the MAPK superfamily include extracellular-regulated kinase (ERK), p38, and the C-Jun N-terminal kinase (JNK). While proliferation and differentiation signals activate ERK, both proliferation and cellular stress signals activate JNK and p38 (1). The JNK signaling pathway is activated by various stimuli including UV light, γ irradiation, osmotic shock, oxidative stress, protein synthesis inhibitors, tumor necrosis factor α, interleukin-1, T-cell costimulatory signals, and mitogenic signals such as Ras (1). JNK activation leads to the phosphorylation of several transcription factors including c-Jun, ATF2, and Elk-1, which in turn increases their transcriptional activity (1).

The JNK signaling pathway is a kinase cascade composed of different levels of MAPKs. Directly upstream of JNK, at the MAPK kinase (MAP2K) level, there are two dual specificity kinases that phosphorylate and activate JNK at serine and threonine residues. These kinases are MAPK kinase 4 (MKK4), and MKK7. These proteins are activated, in turn, by the upstream MAPK kinase kinase (MAP3K): MAPK/ERK kinase (MEKKs), mixed lineage kinase (MLK), TGF-β-activated kinase 1 (TAK1), tumor progression locus 2 (Tpl-2), MAPK upstream kinase (MUK), and apoptosis signal-regulating kinase 1 (ASK1) (3). Recently, a group of MAP4Ks homologous to the Ste20 kinase (an upstream member of the MAPK cascade involved in the pheromone response pathway in Saccharomyces cerevisiae) were identified and characterized (4). These MAP4K proteins provide another level of regulation for the MAPK/JNK signaling cascade and perhaps a link to regulatory proteins that interact with or are located at the plasma membrane.

The MAP4K group includes: hematopoietic progenitor kinase 1 (HPK1) (5, 6), germinal center kinase (GCK) (7, 8), GCK-like kinase (GLK) (9), HPK/GCK-like kinase (HGK) (10), kinase homologous to Ste20/Sps1 (KHS/GCK-related kinase (GCKR)) (11). The murine ortholog of HGK is called Nck-interacting kinase (NIK) (12). Unlike other members of MAP4K group including NIK, HGK does not contain the proline-rich regions. In addition to MAP4K, the p21-activated kinases (PAKs) are another subgroup of the Ste20-like kinases (13). These mammalian Ste20-like kinases all share homology in their kinase domain (3). The PAK kinases contain a Cdc42/Rac1-interactive binding (CIB) domain that allows them to bind to the small GTPases Rac and Cdc42 (13). This binding leads to an increase in the autophosphorylation and, therefore, activation of the PAK kinases (14). However, proteins in the MAP4K subfamily (HPK1, GCK, GLK, HGK/NIK, and KHS/GCKR) do not contain a CIB domain and consequently fail to bind to these regulatory proteins. In particular, the members of the MAP4K subfamily contain a conserved N-terminal kinase domain, a conserved C-terminal tail, and several proline-rich regions in the center of the protein documented to be involved in the association with adaptor proteins.

Hematopoietic progenitor kinase 1 (HPK1) was cloned from a subtractive cDNA library screen between two different progenitor cell libraries (5, 6). HPK1 is a 97-kDa serine/threonine...
kinase belonging to the HPK1/GCK subfamily of protein kinases. HPK1's expression is restricted to adult hematopoietic tissues, and HPK1 protein is also found in hematopoietic cell lines. HPK1 is up-stream of MEKK1 (5) and TGF-β activated kinase 1 (TAK1) (15, 16) in the JNK kinase cascade. HPK1 associates with adaptor proteins such as Crk, CrkL, Grb2, and Nck through binding to the Src-homology domain 3 (SH3) of these proteins (17–19). Furthermore, association of HPK1 with these proteins increases HPK1's kinase activity and its association with the epidermal growth factor receptor (17, 19). It has been demonstrated that the HPK1 proline-rich domains are important for its association with adaptor proteins and its relocation to the plasma membrane where its activity may be regulated.

In this study we describe the cloning of a novel HPK1-interacting protein of 55 kDa (HIP-55), which binds to HPK1. Wild-type HIP-55 showed strong binding to HPK1 in vitro through the second proline-rich domain of HPK1 and in vivo after co-expression. However, a point mutation in HIP-55's SH3 domain abolished this binding to HPK1. Wild-type HIP-55 increased HPK1's kinase activity in co-transfected 293T cells, but the SH3 mutant of HIP-55 did not. The wild-type, but not mutant, form of HIP-55 also increased JNK's kinase activity, a phenomenon that could be specifically blocked by a dominant-negative HPK1 mutant. Collectively, we have identified a novel protein that activates the JNK signaling pathway through HPK1.

EXPERIMENTAL PROCEDURES

Plasmid Construction for the Yeast Two-Hybrid System and Yeast Two-Hybrid Library Screen—Full-length GLK cDNA (9) was subcloned into yeast plasmid pGBT9 (CLONTECH) to create an in-frame fusion with the DNA-binding domain gene. The pGBT9-GLK was transformed into yeast strain HF7c using the lithium acetate procedure and plated onto synthetic complete (SC) media lacking tryptophan. Plasmid DNA from human HeLa cell cDNA library (CLONTECH) was then transformed into the yeast strain containing the GLK bait plasmid and plated on SC medium minus tryptophan, leucine, and histidine and grown at 30 °C for 3–5 days. Transformants were assayed for β-galactosidase activity. Library plasmid DNA was recovered by transformation into DH10B cells and sequenced on both strands.

Plasmid Construction—Full-length HIP-55 was cloned into mammalian expression vectors PCRS.1 (Invitrogen, San Diego, CA) by PCR using two oligonucleotide primers. The oligonucleotides used were the following: 5'-TACGGTTGGCCGGCCCGCCAGTCGAGCGGAACGGGAAA- C-3' and 5'-AGCGCCGGCCCGCGGCTCCAGCTACTCAGTACGCGC-3'. PCR products were cut with SfiI and NotI and cloned into a pME- vector with an in-frame hemagglutinin (HA)-epitope sequence at the 5' end. For construction of glutathione S-transferase (GST)-HIP-55 protein, HIP-55 was subcloned into pGEX4T-3 vector. A tryptophan mutant in the SH3 domain was generated by replacing residue 408 with a glutamic acid and sequenced on both strands. For co-expression, the cells were harvested and lysed in lysis buffer (500 mM LiCl, 100 mM Tris-Cl, pH 7.6, 0.1% Triton X-100). Proteins were separated by SDS-PAGE as described previously (10) and immunoblotted using an anti-FLAG antibody (M2) (Eastman Kodak Co.,) and visualized by chemiluminescence (ECL, Amersham Pharmacia Biotech). For co-immunoprecipitation, 500 μg of transfected 293T lysate was used with 3 μl of an anti-HA monoclonal antibody (12CA5, Roche Molecular Biochemicals) as described previously (17).

Peptide Competition Assays—[35S]Methionine-labeled HIP-55 protein was generated by in vitro transcription and translation (Promega Biotech, Inc.) following manufacturer's instructions. For the peptide competition assay, 1 μl peptides (previously described in Ref. 17) corresponding to HPK1's proline-rich domains were used in the same GST-HIP-55 in vitro binding conditions as described previously except for the presence of [35S]methionine-labeled HIP-55 instead of cell lysate. The protein complexes were washed and separated on SDS-PAGE, and the radiolabeled protein was visualized by autoradiography.

RESULTS

Molecular Cloning of HIP-55—The yeast two-hybrid system was used to identify proteins that interact with GLK. Several clones were identified and sequenced. Two clones (F1 and F2) appeared to contain novel cDNA sequences and were derived from the same gene. The cDNA was then transformed into yeast strain HF7c along with either GLK or HIP1 bait plasmids or several other bait plasmids. F1 and F2 were found specifically to interact with HPK1 and GLK but not with other kinases such as MK66 or MAPKKK5 (data not shown). To isolate a full-length cDNA clone of F1 and F2, we searched the EST data base with F1 and F2 sequences for additional 5' end sequence. Overlapping fragments were identified that contained an initiation codon followed by stop codons. These EST clones were obtained and sequenced on both strands. Primers were then synthesized based on the EST sequence and 5' end sequence of the F1 and F2 clones and used to amplify the full-length cDNA. The complete nucleotide sequence of the cDNA predicted an open reading frame of 430 amino acids with a predicted molecular mass of 48 kDa (Fig. 1A). Further characterization of this protein showed an apparent molecular mass of 55 kDa by SDS-PAGE. We therefore designated the novel molecule as HIP-55, HIPK1-interacting protein of 55 kDa. Data base searches found that the N-terminal of HIP-55 contains a proline-rich domain that is found in drebrins (21), protein A-Sepharose column.
translated product was detected by an anti-HA antibody (Fig. 2A) and an anti-HIP-55 antibody (Fig. 2B). To examine the endogenous HIP-55 protein expression, we used various cell lysates from 293T, HeLa, HL-60, and Jurkat (data not shown) cell lines (Fig. 2B). Protein lysates from these cells were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and blotted with a purified anti-HIP-55 antibody. The HIP-55 proteins were expressed in all of the cell lines tested, and the expression levels appear to be high and similar between the various cell lines (Fig. 2B). As mentioned previously, the HIP-55 protein had an apparent molecular mass of 55 kDa, and was accordingly named HIP-55. Northern blot analysis of HIP-55 showed a single transcript of ~2.3 kilobases in all the tissues studied, indicating that HIP-55 mRNA is ubiquitously expressed (Fig. 2C). The level of HIP-55 mRNA expression appeared to be higher in the spleen and peripheral blood leukocytes when compared with other tissues.

HIP-55 Bound to HPK1 in Vitro—In order to confirm the binding results from the yeast two-hybrid system, we examined the in vitro binding of HIP-55 to HPK1. We chose to focus on HPK1, rather than GLK, since more is known about HPK1 and its interactions with other adaptor proteins including Grb2, Crk, CrkL, and Nck (17). GST-HIP-55 was constructed and expressed in *Escherichia coli*. The expressed proteins were then affinity-purified by glutathione-Sepharose beads. Lysate from 293T cells transfected with FLAG-HPK1 was incubated with the immobilized GST-HIP-55 protein. The protein complex was washed extensively, separated by SDS-PAGE, and transferred to a polyvinylidene difluoride membrane, and the membrane was blotted using anti-FLAG antibody. Transfected HPK1 associated with GST-HIP-55, but not with the GST protein. In addition, no unspecific associations with GST-HIP-55 were detected in the vector-transfected lane (Fig. 3A).

HPK1 Associated with HIP-55 in Vivo after Co-expression—To analyze the in vivo binding of HPK1 and HIP-55, we co-transfected these two plasmids or HPK1 and the HIP-55 SH3 mutant (W408K) into 293T cells. The cells were lysed 36 h after transfection, and HIP-55 was immunoprecipitated, using an anti-HA antibody. The protein complexes were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane that was subsequently blotted with an anti-FLAG antibody to detect FLAG-HPK1. HPK1 protein co-immunoprecipitated with the wild-type HIP-55 protein but failed to bind to the HIP-55 SH3 mutant (Fig. 3B). This indicates that HIP-55 interacts with HPK1 in vivo, and this interaction is mediated through HIP-55’s SH3 domain.

HPK1 Binds HIP-55 through Its Second Proline-rich Domain—We further analyzed HPK1’s proline-rich domains to identify the one(s) involved in this binding. We performed the in vitro binding assays of GST-HIP-55 and HPK1 using [35S]methionine in vitro translated and labeled HPK1 protein. To compete for binding to HPK1, we also included synthetic peptides corresponding to HPK1’s four proline-rich domains (PR1–PR4) (Fig. 3C). The protein complexes were washed and separated by SDS-PAGE, and the presence of radioactive HPK1 was determined by autoradiography. Only the addition of a peptide corresponding to HPK1’s second proline-rich domain (PR2) weakened the interaction between HPK1 and GST-HIP-55 (Fig. 3C). None of the other peptides appeared to diminish the binding between HIP-55 and HPK1. These results suggest that HPK1 binds to HIP-55 through a proline-rich domain/SH3 interaction that involves HPK1’s second proline-rich domain.

Quantitative Analysis of HPK1 and HIP-55 Interaction—More detailed analysis of the HPK1 and HIP-55 binding was carried out using immunodepletion studies. The total percentage of HIP-55 bound to HPK1 could not be studied owing to the
co-migration of HIP-55 with the immunoglobulin heavy chain during immunoprecipitation. However, about 58% of HIP-55 was in the free or unbound form following HIPK1 depletion from the lysate (Fig. 4A), suggesting that the remaining 42% was bound to HIPK1. Furthermore, 22% of total HIPK1 bound to HIP-55 and the remaining 78% was in the free or unbound form (Fig. 4B).

HIP-55 Increased HIPK1's Kinase Activity—To determine the effect of HIP-55 binding to HIPK1, we analyzed HIPK1's kinase activity from cells co-transfected with HIP-55. 293T cells were transfected with FLAG-HIPK1 and HA-HIP-55 alone or in combination. HIPK1 was immunoprecipitated from the cell lysates and incubated in a kinase reaction with myelin basic protein as a substrate. Co-transfection of HIP-55 with HIPK1 resulted in an increase in HIPK1's kinase activity in vitro (Fig. 5A). These results suggest that HIP-55 not only binds to HIPK1 but may also be involved in the regulation of HIPK1's kinase activity.

HIP-55 Activates JNK1 through HIPK1—Since wild-type
HIP-55 bound to and activated HPK1, an upstream regulator of the JNK1 signaling pathway, we analyzed whether HIP-55 could activate JNK. 293T cells were transiently transfected with HIP-55 wild-type or the SH3 mutant in addition to HA-JNK1. JNK1 kinase assays showed that the wild-type HIP-55 could activate the MAPK while the SH3 mutant failed to do so (Fig. 5B). To examine if HPK1 was mediating the activation of JNK1 by HIP-55, we added a dominant-negative mutant of HPK1, HPK1-M46 (5), to the transfections. The presence of the HIP-55 mutant completely blocked activation of JNK1 by HIP-55, indicating that HIPK1 kinase mediates the activation of JNK1 by HIP-55 (Fig. 5B). Furthermore, HKG-KE (10), a dominant-negative HGK mutant, did not block HIP-55 induced JNK1 activation. Additional in vitro binding assays using GST-HIP-55 showed that it did not bind to the HGK protein, which lacks the proline-rich domains (data not shown). These results indicate that HIP-55 activates JNK1 through HPK1 and this is mediated by binding of HPK1 to HIP-55.

**DISCUSSION**

We have cloned a novel protein, HIP-55, that bound to HPK1. From the two-hybrid system and the in vitro competition binding assays, we found that HPK1 bound to HIP-55 through its second proline-rich domain. We also show that HPK1 and HIP-55 are capable of interacting with each other in 293T cells. Our studies show that the presence of HIP-55 in HPK1-transfected lysate increases HPK1's kinase activity, suggesting that the interaction between these two proteins is functionally relevant in cells. We detected a reproducible increase in HPK1 protein levels when co-transfected with wild type HIP-55. This increase in HPK1 protein levels was not seen in co-transfection assays with a SH3 mutant of HIP-55, indicating that interaction with HPK1 is required for the increase in protein levels, and hence HPK1 kinase activity. In comparison, HPK-KD protein levels (and kinase activity) did not change when co-transfected with HIP-55 (data not shown). HPK-KD is in the same vector as wild type HPK1, thus eliminating any effect of HIP-55 on transcriptional up-regulation of the cytomegalovirus promoter-driven HPK1 expression. Furthermore, HPK-KD does not contain the proline-rich domains found on wild type HPK1. This emphasizes the importance of binding of HIP-55 to HIP-55 for increases in HPK1 kinase activity and protein levels. We also found that p38 protein levels did not increase when co-transfected with HIP-55 (data not shown), indicating that the increase in protein levels is not a general effect of HIP-55. Our observations suggest that HIP-55 specifically activates HPK1 and this is in part through increasing HPK1 protein levels. We are currently pursuing the mechanisms by which HIP-55 may lead to increases in HPK1 protein levels.

HIP-55 increases JNK1's kinase activity, and the activation of JNK1 is mediated by HPK1 since it can be blocked by a dominant-negative HPK1 mutant. Therefore, HIP-55 acts as an upstream activator of HPK1 and the JNK1 signaling pathway. We also showed that HIP-55's SH3 domain is critical for its effect on kinase activity of HPK1 and JNK1 since mutated HIP-55 failed to bind to HPK1 and also failed to activate HPK1 and JNK1. This result suggests that binding of the HIP-55 SH3 domain to HPK1 is required for the increase on HPK1's kinase activity. We are actively studying the detailed mechanism by which HIP-55 leads to HPK1 activation.

HIP-55 homology searches (BLAST, FASTA) identified several proteins that shared homology to HIP-55. Three actin-binding proteins were identified: drehrin, an actin-binding protein expressed in brain tissue and neurons (21); actin-binding protein 1 (Abp1), an S. cerevisiae protein involved in spatial organization of cell surface growth (22), and coactosin, a Dictyostelium discoideum protein known to bind actin filaments (23). More detailed analysis of these proteins showed that the actin-binding domains of drehrin, Abp1, and coactosin were homologous (36%, 36%, and 21%, respectively) with the N terminus of HIP-55 (Fig. 1B). In addition to our homology search results, we found a mouse clone named SH3P7 that was isolated in a screen conducted to identify SH3 domain-containing proteins (24). SH3P7 is 85% identical to HIP-55 at the amino acid level. We therefore suspect SH3P7 may be the mouse ortholog of HIP-55. Interestingly, SH3P7 was recently classified as an actin-binding protein containing an actin-depolymerizing factor (ADF) domain and grouped with drehrin and Abp1 (25, 26). HIP-55 (and SH3P7) retains all of the residues shown important for actin binding in yeast cofilin as well as the secondary structural elements (as derived from models) of the ADF domain. Furthermore, SH3P7 is capable of binding to actin filaments (25). It is very likely, therefore, that HIP-55 protein will also bind to actin filaments. This possibility suggests a novel mechanism for the regulation of MAP4Ks through interaction with HIP-55 and the cytoskeleton. However, this prospect remains to be explored.
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