Identification and Characterization of RPK118, a Novel Sphingosine Kinase-1-binding Protein

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Sphingosine kinase (SPHK) is a key enzyme catalyzing the formation of sphingosine 1 phosphate (SPP), a lipid messenger that is implicated in the regulation of a wide variety of important cellular events through intracellular as well as extracellular mechanisms. However, the molecular mechanism of the intracellular actions of SPP remains unclear. Here we have cloned a novel sphingosine kinase-1 (SPHK1)-binding protein, RPK118, by yeast two-hybrid screening. RPK118 contains several functional domains whose sequences are homologous to other known proteins including the phox homology domain and pseudokinase 1 and 2 domains and is shown to be a member of an evolutionarily highly conserved gene family. The pseudokinase 2 domain of RPK118 is responsible for SPHK1 binding as judged by yeast two-hybrid screening and immunoprecipitation studies. RPK118 is also shown to co-localize with SPHK1 on early endosomes in COS7 cells expressing both recombinant proteins. Furthermore, RPK118 specifically binds to phosphatidylinositol 3-phosphate. These results strongly suggest that RPK118 is a novel SPHK1-binding protein that may be involved in transmitting SPP-mediated signaling into the cell.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening and cDNA Cloning—Full-length mouse sphingosine kinase-1α cDNA (DBJ/EMBL/GenBank™ accession no. AF068748) was cloned into pGBK7 (CLONTECH) in-frame with the GAL4 DNA-binding domain. The bait was transformed into the yeast strain AH109 together with the rat brain cDNA library (CLONTECH) fused to the GAL4 activation domain in the pGADT7 according to the manufacturer's instructions (CLONTECH). DNA from positive clones was prepared from yeast and transformed into competent DH5α (TaKaRa, Otsu, Japan) according to standard protocols. From partial RPK118 cDNA sequence information, a complete human RPK118 cDNA was obtained (DBJ/EMBL/GenBank™ accession no. AB070706) by using a 5'-rapid amplification of cDNA ends (5'-RACE) method as described (10) with the total human brain cDNA reverse transcribed from fetal human brain mRNA (Invitrogen).

Mammalian Expression Vectors—The full-length RPK118, a PSK2 fragment, or RPK1ΔPSK were each subcloned into mammalian expression vector pCMV5 with a FLAG-epitope tag to express N-terminal FLAG-tagged fusion proteins in mammalian cells. RPK118 was also cloned into pEGFP-C1 (CLONTECH) for N-terminal FLAG-tagged fusion proteins in mammalian cells.

Immunoprecipitation—COS7 cells transiently expressing both FLAG-RPK118 and influenza hemagglutinin (HA)-SPHK1 were lysed in cold lysis buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1% (w/v) Brij 35, 20 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, 0.2 mM dithiothreitol) and cleared by centrifugation for 15 min at 10,000 × g.
97 (Sigma) and protease inhibitors (Roche Molecular Biochemicals)). Immunoprecipitation and immunoblot analyses were carried out as described (10) using the anti-FLAG antibody M2 (Sigma) and the anti-HA antibody (Roche).

Interaction of RPK118 with SPHK1—COS7 cells expressing FLAG-RPK118 or FLAG-ribosomal S6 kinase 3 (RSK3) were lysed in cold lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Nonidet P-40, 0.5% Triton X-100, 1 mM Na3VO4, and protease inhibitors). FLAG-tagged proteins were immunoprecipitated using anti-FLAG antibody M2 beads. The immunoprecipitates were washed and measured for in vitro protein kinase activity without or with 250 μM ATP (Upstate Biotechnology) as described (11). The incorporation of 32P into the S6 peptide was determined by SDS-PAGE followed by quantification using a Fujix Bio-Imaging Analyzer BAS2000 (Fuji Photo Film).

Northern Blotting Analysis—Poly(A) RNA blot containing 1 μg of poly(A) RNA per lane from multiple human tissues (CLONTECH) were hybridized with the 32P-labeled 900-bp BamHI fragment of pCMV-human RPK118. Hybridization was carried out according to the manufacturer’s protocol. Bands were visualized using a Fujix Bio-Imaging Analyzer BAS2000.

Confocal Microscopy—Transfected COS7 cells were seeded on cover slips (Naige Nunc) at a density of 106 cells ml⁻¹, fixed with 3% paraformaldehyde in phosphate-buffered saline for 15 min, permeabilized with 0.2% Triton X-100 for 10 min, and blocked with 0.5% bovine serum albumin in phosphate-buffered saline containing 1% bovine serum albumin. The cells were incubated with primary antibodies for 1 h at 25 °C, washed three times, and incubated with fluorochrome-conjugated secondary antibodies for 1 h at 25 °C. Microscopy was performed with a confocal microscope (Bio-Rad MRC1024). For double staining, control scabs confirmed that no bleed-through was detectable under the condition used. No signal was obtained if the first antibody was omitted. Alexa 488-conjugated goat anti-mouse IgG and Alexa 594-conjugated goat anti-rat or anti-mouse IgG were from Molecular Probes (Eugene, Oregon). The early endosomal antigen 1 (EEA1) antibody was from BD Transduction Laboratories (San Jose, CA).

Dot-blot Overlay Assay—Recombinant glutathione S-transferase (GST)-RPK118 generated in the S9 cell expression system as described previously (10) was further purified by glutathione-Sepharose 4B (Amersham Biosciences). The purified GST-RPK118 was used to probe bovine serum albumin-blocked nitrocellulose filters (Echelon Research Laboratories, Salt Lake City, UT) on which various phospholipids had been spotted. The filters were washed, and GST-RPK118 bound to the filters was detected using the anti-GST antibody (Amersham Biosciences).

RESULTS

Identification of a Novel SPHK1-interacting Molecule, RPK118, with the PX, EPS, PSK1, and PSK2 Domains—In an effort to identify proteins that may participate in the recruitment of SPHK to the right destination where intracellular SPP will accumulate, we conducted a yeast two-hybrid screen of a rat brain cDNA library using full-length mouse SPHK1 cDNA as bait. Four independent clones encoding amino acid sequences almost identical to the C-terminal region of a previously reported “human ribosomal S6 kinase” gene product (RSK52) with a calculated molecular mass of 52kDa (12) were isolated (Fig. 1A). We assumed that the four clones are parts of a rat homologue of human RSK52. We then isolated the human homologous (PX) domain (13), a Saccharomyces cerevisiae phox homology (PX) domain (14), and pseudokinase 1 (PSK1) and 2 (PSK2) domains (Fig. 2, and see below). The PX domain, the function of which is as yet unknown, is an evolutionarily conserved sequence that is present in a number of proteins with diverse functions, including proteins involved in vesicular trafficking (15–18). The ESP domain, whose function is also unclear at present, may be involved in the association of the Vps4 protein with endosomal membranes (19). The C-terminal half of RPK118 contains two conserved sequences arranged in tandem that show homology to the regions essential for the catalytic activity of RSK3 (20). The first sequence (residues 426–437) corresponds to the kinase subdomains I to V (Fig. 3A), whereas the second sequence (residues 906–1066) corresponds to the kinase subdomains VI to X (Fig. 3B) with a large unrelated insert between the sequences (Fig. 1B). The GXXGXX motif essential for ATP binding in the first sequence, which corresponds to the kinase subdomain I of RSK3, and the DFG motif important for conferring Mg2⁺ sensitivity to the enzyme in the second sequence, which corresponds to the kinase subdomain VII of RSK3, were both mutated as shown in Fig. 3, A and B, suggesting that the protein is defective in
phosphotransferase activity. Indeed, the immunoprecipitated 118-kDa protein showed no kinase activity toward either itself or the exogenous substrate despite good expression of the 118-kDa protein and fair kinase activity of RSK3 (Fig. 4, A and B), confirming its structural parameters (Fig. 3). Therefore, we have designated these regions of homology as the PSK1 and PSK2 domains, respectively, after pseudo-kinase. We have also termed this protein as RPK118 after the ribosomal S6 kinase-like protein with two PSK domains. A database search identified orthologues of RPK118 in Drosophila melanogaster and Caenorhabditis elegans, demonstrating that RPK118 is a member of a novel and highly conserved gene family (Fig. 2). These cDNA sequences were from putative open reading frames identified from D. melanogaster and C. elegans. A comparison of these sequences reveals the presence of all characteristic homology domains including the PX, ESP, PSK1, and PSK2 domains. There also exists a human gene encoding a putative protein, RPK60 (with the ESP, PSK1, and PSK2 domains but devoid of a PX domain), which was originally reported as a “unknown kinase” (GenBank™ accession no. AAD30182) and whose function is currently unknown.

**Biochemical Characterization of RPK118**—The tissue distribution of RPK118 mRNA in human tissues was analyzed by Northern blotting (Fig. 5). RPK118 was ubiquitously distributed among various tissues tested with the highest levels of mRNA detected in the skeletal muscle, brain, heart, placenta, kidney, and liver. Next, the binding results obtained from the yeast two-hybrid screening were further confirmed by documenting the interaction between RPK118 and SPHK1 directly. We conducted immunoprecipitation experiments using FLAG-RPK118, FLAG-PSK2 fragment, another deletion mutant FLAG-RPK/H9004 that is devoid of the C-terminal half of the sequence (from residues 314 to 1066, including the PSK1 and PSK2 domains), and HA-SPHK1. These epitope-tagged proteins were expressed in COS7 cells, and the interaction of these proteins was analyzed. HA-SPHK1 was specifically co-immunoprecipitated with FLAG-RPK118 (Fig. 6). The FLAG-PSK2 fragment also interacted with SPHK1, confirming the results from yeast two-hybrid analyses that show that the binding site for SPHK1 is localized within the PSK2 domain of RPK118 (Fig. 1A). FLAG-RPKΔPSK showed no interaction with SPHK1.

**RPK118 Co-localizes with SPHK1 in COS7 Cells**—We investigated the interaction of RPK118 with SPHK1 in intact cells...
using immunofluorescence techniques. For the characterization of subcellular localization of RPK118, we constructed RPK118 fused with GFP. The utility of GFP-RPK118 was verified by demonstrating co-localization of GFP-RPK118 and FLAG-RPK118 in COS7 cells expressing both proteins. These proteins distributed diffusely in the cytosol and in some small dot-like or ring-shaped structures where the proteins showed exact co-localization (Fig. 7, A–C, arrows). In contrast, GFP itself was distributed diffusely throughout COS7 cells express-
ing GFP vector alone (data not shown). When EEA1, an endogenous marker of early endosomes, was immunostained, EEA1 displayed good co-localization with GFP-RPK118 (Fig. 7, D–F, arrows), indicating that the RPK118-positive dot-like or ring-shaped structures were in fact early endosomes. Next, when GFP-RPK118 and HA-SHPK1 were expressed simultaneously in COS7 cells, GFP-RPK118 was distributed diffusely in the cytoplasm (except nuclei) and also in putative early endosomes (Fig. 7G). A similar pattern was observed when this protein alone was expressed in COS7 cells (Fig. 7, A–D), whereas HA-SHPK1 was distributed in a fine reticular pattern in the cytoplasm with early endosomal distribution (Fig. 7H). Some but not all endosomal structures were co-labeled both with GFP-RPK118 and HA-SHPK1 (Fig. 7I, arrows). The dot-like or ring-shaped endosomal localization pattern of RPK118 may not be a consequence of high levels of the protein expression, because this pattern was also observed in the cells expressing the protein at a relatively low level (data not shown). Interestingly, in the cells expressing HA-SHPK1 but not GFP-RPK118 (Fig. 7H, arrowhead), HA-SHPK1 staining with putative early endosomal structures was hardly observed. To demonstrate the direct involvement of RPK118 in the recruitment of SHPK1 to the early endosomes, the effect of PSK2 (an SHPK1-binding fragment of RPK118 as suggested by Figs. 1A and 6) on SHPK1 distribution was tested. When the PSK2 fragment was expressed together with GFP-RPK118 and HA-SHPK1 in COS7 cells, HA-SHPK1 was distributed both in the cytoplasm and the peripheral area without any endosomal labeling (Fig. 7L), leaving the staining pattern of GFP-RPK118 almost unchanged (Fig. 7K). Thus, the PSK2 fragment may function as a dominant negative by competing with RPK118 for SHPK1 binding. These results clearly demonstrate the importance of RPK118 in the recruitment of SHPK1 to early endosomes.

**RPK118 Interacts Specifically with Phosphatidylinositol 3-Phosphate (PtdIns (3)P) through Its PX Domain**—Recent observations that proteins containing the PX domain specifically recognize PtdIns (3)P at specific membrane surfaces (15–18, 21) prompted us to ask whether RPK118 also binds to PtdIns (3)P through its PX domain. To study the phosphoinositide binding specificities of RPK118, we employed a dot-blot overlay assay. As shown in Fig. 8, RPK118 exhibited specific binding to PtdIns (3)P with other phosphoinositides. RPK118 interacted weakly with phosphatidylinositol 5-phosphate but not at all with phosphatidylinositol 4,5-bisphosphate or phosphatidylinositol 3,4,5-trisphosphate. A deletion mutant, RPK118ΔPX, which lacks the PX domain of RPK118, failed to bind to PtdIns (3)P on the filters (data not shown).

**DISCUSSION**

We have shown here that a newly identified protein, RPK118, can bind to and co-localize with SHPK1 in COS7-cells. The PSK2 domain of RPK118 is required for binding to SHPK1 as demonstrated by the results from immunoprecipitation analyses (Fig. 6) as well as yeast two-hybrid screening (Fig. 1A). The overexpression of RPK118 in COS7 cells did not cause any change in the intracellular content of SPP with repeated experiments, and RPK118 binding to SHPK1 did not alter the enzymatic activity of SHPK1 in vitro (data not shown), suggesting that RPK118 may function only as an adaptor molecule for SHPK1.

Analysis of the subcellular distribution of SHPK will provide important clues to the understanding of the mechanism of intracellular action of SPP. It has been reported (22) that SHPK1 expressed in HEK293 cells was detected in both cytosol and membrane fractions. More detailed subcellular studies using density gradient centrifugation have also shown the membrane-associated SHPK activities, especially in vesicles derived from the endoplasmic reticulum and the plasma membrane in rat tissues (23). Our present studies indicate that SHPK1 distributes in a fine reticular pattern in the cytoplasm and that it co-localizes with RPK118-positive ring-shaped early endosomes when RPK118 is co-expressed with SHPK1 (Fig. 7, G–I). We also show that the ring-shaped endosomal pattern of SHPK1 distribution was completely altered to a nearly homogeneously diffuse pattern by the expression of the PSK2 fragment, the SHPK1-binding site of RPK118 (compare Fig. 7, H and I). This suggests that the intracellular localization of SHPK1 may vary depending on the functional state of the cell. Indeed, while this manuscript was under review, Melendez and Khaw (24) reported that antigen stimulation of human mast cells induced a rapid translocation of SHPK1 from the cytosol to the "nuclear-free membrane fractions." Our present results strongly suggest that RPK118 may at least in part determine the endosomal localization of SHPK1. The mechanism of stimulation-induced translocation of SHPK1 to the appropriate cellular destination through RPK118 remains to be elucidated. RPK118-positive ring-shaped structures putatively identified as early endosomes based on EEA1 labeling were also co-labeled with the early recycling endosomal marker Rab4 (data not shown). Further studies are necessary for understanding the physiological relevance of SHPK1 recruitment to early endosomes by RPK118.

From the structural analysis, it is obvious that RPK118 has the highest sequence homology with sorting nexin 15 (SNX15) (14). Sorting nexins are an emerging family of proteins with a PX domain that are involved in regulating vesicular transport (13, 14, 25, 26). The sequence of the PX domain of human SNX15 is 76.5% similar (55.3% identical) to that of RPK118. SNX15 also contains ESP but not PSK domains where SHPK1 binds. The exact destination where the PX domains of RPK118 as well as SNX15 bind remains to be identified. Recently, Xia et al. (27) reported that SHPK interacts with tumor necrosis factor-α receptor-associated factor 2 (TRAF2). However, there is no sequence homology between RPK118 and TRAF2.

PtdIns (3)P has been implicated in the regulation of endosomal traffic because the yeast Vps34p, a yeast protein essential for protein targeting to the yeast vacuole and vacuole morpho-
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