Protein Kinase D-mediated Phosphorylation and Nuclear Export of Sphingosine Kinase 2*

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Sphingosine kinase (SPHK) is a key enzyme producing important messenger sphingosine 1-phosphate and is implicated in cell proliferation and suppression of apoptosis. Because the extent of agonist-induced activation of SPHK is modest, signaling via SPHK may be regulated through its localization at specific intracellular sites. Although the SPHK1 isoform has been extensively studied and characterized, the regulation of expression and function of the other isoform, SPHK2, remain largely unexplored. Here we describe an important post-translational modification, namely, phosphorylation of SPHK2 catalyzed by protein kinase D (PKD), which regulates its localization. Upon stimulation of HeLa cells by tumor promoter phorbol 12-myristate 13-acetate, a serine residue in a novel and putative nuclear export signal, identified for the first time, in SPHK2 was phosphorylated followed by SPHK2 export from the nucleus. Constitutively active PKD phosphorylated this serine residue in the nuclear export signal both in vivo and in vitro. Moreover, down-regulation of PKDs through RNA interference resulted in the attenuation of both basal and phorbol 12-myristate 13-acetate-induced phosphorylation, which was followed by the accumulation of SPHK2 in the nucleus in a manner rescued by PKD over-expression. These results indicate that PKD is a physiologically relevant enzyme for SPHK2 phosphorylation, which leads to its nuclear export for subsequent cellular signaling.

Sphingosine kinases (SPHKs) catalyze the formation of sphingosine 1-phosphate, a bioactive lipid that regulates a diverse range of cellular processes, including cell growth, survival, differentiation, motility, and cytoskeletal organization (1, 2). Some of these cellular processes are mediated by five sphingosine 1-phosphate-specific G protein-coupled receptors, whereas others appear to be controlled by intracellular sphingosine 1-phosphate through as yet unidentified intracellular targets (2, 3).

Two distinct SPHK isoforms, SPHK1 and SPHK2, have been cloned and characterized (4, 5). Diverse external stimuli, particularly growth and survival factors, stimulate SPHK1, and intracellularly generated sphingosine 1-phosphate has been implicated in their mitogenic and anti-apoptotic effects (6–15). Expression of SPHK1 enhanced proliferation, promoted the G1/S transition, protected cells from apoptosis (6, 8, 16), and induced tumor formation in mice (8, 9).

In contrast to SPHK1 much less is known about SPHK2. Although highly similar in amino acid sequence and possessing five evolutionarily conserved domains found in all SPHKs (17), SPHK2 diverges in its N terminus and central regions. These two isoforms have different kinetic properties and differ in developmental and tissue expression (5) implying that they may have distinct physiological functions. In fact, studies from our laboratory have demonstrated that, in contrast to cytosolic distribution of SPHK1, SPHK2 enters nuclei and inhibits DNA synthesis or induces apoptosis under stressful conditions such as serum deprivation (18, 19). Similarly, Liu et al. have reported that SPHK2 induces apoptosis through its putative BH3 domain (20). More recently, SPHK2 has been shown to phosphorylate an immunosuppressant drug FTY720 (21, 22) and, further, to associate with interleukin-12 receptor in T cells (23), suggesting a potential immunomodulatory action. However, the physiological role of SPHK2 remains largely unknown.

Nucleo/cytoplasmic shuttling of protein and RNA molecules plays an important role in eukaryotic cell function (24). A related family of shuttling transport factors, importins and exportins, recognize nuclear localization signal (NLS)-containing or nuclear export signal (NES)-containing proteins. The coordination of trafficking between the nucleus and the cytoplasm is determined by the balance of nuclear import and export activity (25–27), which is specifically regulated by various physiological or environmental determinants. Modification of proteins regulates their function, location, and stability. The present studies were undertaken to investigate the post-translational phosphorylation of SPHK2 and its involvement in the regulation of nucleo/cytoplasmic shuttling of this protein. Here we report, for the first time, that SPHK2 can be phosphorylated in an agonist-dependent manner. We also have identified a unique NES, in SPHK2, whose function is...
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regulated by protein phosphorylation. Furthermore we have identified the kinase responsible for the phosphorylation of the serine residue in the NES as protein kinase D (PKD) and demonstrate that the phosphorylation of SPHK2 by PKD is critical for its nuclear export under physiological conditions.

EXPERIMENTAL PROCEDURES

Plasmid Construction—N-terminally influenza hemagglutinin (HA)-tagged human SPHK2-L (hSPHK2-L) was constructed as described (19) and cloned into pEGFP-N1 (Clontech, Palo Alto, CA). hPKD1 (GenBank™ accession number, X75756), hPKD2 (GenBank™ accession number, AF309082), and hPKD3 (GenBank™ accession number, AB015982) cDNAs were amplified from a human brain cDNA (hPKD1 and hPKD2) or human ovary cDNA (hPKD3), by PCR (sense primer, 5′-CGC AGG ATG AGC GCC CCT CGG GTC CTG CGG-3′ and antisense primer, 5′-AGA ATC GAT TCA GAG GAT GCT GAC GCT ACC-3′, for hPKD1; sense primer, 5′-CCG AGG ATG AGC ACC GCC CCC CTCT TAT CCC-3′ and antisense primer, 5′-CAC ATC GAT TCA GAG AAC ACT GAT GCG CTC GCC-3′, for hPKD2; sense primer, 5′-GAA AGC AGT ATG TCT GCA AAT AAT TCC CCT CCA-3′ and antisense primer, 5′-GCT ATC GAT TTA AGG ATC TTC TTC CAT ATC ATC-3′, for hPKD3) to make N-terminally HA-tagged constructs.

Site-directed mutagenesis was performed using the Stratagene (La Jolla, CA) QuikChange site-directed mutagenesis kit to prepare various mutants. The primers used were as follows: putative NES mutant (S419A/S421A), 5′-GCA GGG GAG CAG GCG CGT CAG ACA CAG AACG-3′; putative NES mutant (L306A/L308A), 5′-GTC AGT GGT-3′; putative NES mutant (L306A/L308A), 5′-ACC ATC GAT TCA GAG AAC ACT GAT GCG CTC GCC-3′, for hPKD2; sense primer, 5′-GAA AGC AGT ATG TCT GCA AAT AAT TCC CCT CCA-3′; and antisense primer, 5′-GCT ATC GAT TTA AGG ATC TTC TTC CAT ATC ATC-3′, for hPKD3) to make N-terminally HA-tagged constructs.

PKD Preparation—COS7 cells were transfected with HA-tagged constitutively active PKD3 or kinase-negative PKD3. Three days after transfection cells were washed three times in ice-cold phosphate-buffered saline and lysed in the lysis buffer (50 mM Tris-HCl at pH 7.6, 2 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol, 10 μg/ml leupeptin, 15 mM NaF, 30 mM β-glycerophosphate, 1 mM Na3VO4, protease inhibitor mix (Roche Applied Science), and 1% Triton X-100). PKD was immunoprecipitated for 4 h at 4 °C with the anti-HA affinity matrix (Roche Applied Science) and then eluted for 12 h at 4 °C with HA-peptide (Sigma, 100 μg/ml) in kinase buffer (30 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 1 mM dithiothreitol).

Peptide Phosphorylation by PKD—Peptide phosphorylation by PKD was determined as described previously (28). Briefly, 2.5 mg/ml syntide-2 or the NES peptide was phosphorylated by affinity-purified PKD in the presence of 100 μM [γ-32P]ATP and 10 mM MgCl2. After incubation at 30 °C, the reaction was terminated by adding 75 mM H3PO4 and spotting onto P-81 phosphocellulose paper (Millipore). After washing the paper with 75 mM H3PO4, the radioactivity incorporated into peptides was determined by Cerenkov counting.

Cell Cultures—HeLa and HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO2 at 37 °C.

siRNA—siRNA for human PKD1 (5′-CAAGUCAAUCUUGUUGUGUUGAAT-3′ and 5′-UCAACGAAUAGGGGUUCGCCG-3′), human PKD2 (5′-CAAGUUAUCUCAUCAAGAGGUCGCCGACG-3′ and 5′-UUUGUGACAACUUAAAGCUAAGC-3′), human PKD3 (5′-CGCGUACCAAGAGGUGACACT-3′ and 5′-UAAGUUCGUUUGGCCAGACG-3′), and the control siRNA (5′-UUCUCCGAGCGUUGCAGUACUCAA-3′ and 5′-UACUUAGGUUCGCGAGGACG-3′) were synthesized at Japan Bio Services (Saitama, Japan). HeLa cells were transfected both with siRNA and various expression vector constructs using Lipofectamine2000.

Antibody—Preparation of an antibody against hSPHK2 has been described (18). Phosphorylated SPHK2 (P-SPHK2)-specific polyclonal antibody was raised in rabbits against a phosphopeptide (CSPLHRpSVpSDLPLP) encompassing the NES region fused with keyhole limpet hemocyanin. Nonspecific antibodies were removed by passing through a HitTrap N-hydroxysuccinimide-activated HP column (GE Healthcare Bio-Science Corp.) conjugated with the corresponding non-phosphorylated peptide (CSPLHRpSVpSDLPLP). This antibody can be used for immunoblotting and immunohistochemistry but not for immunoprecipitation studies.

For the detection of endogenous P-SPHK2 HepG2 cell lysates (500 μg of protein) were mixed with 30 μl (gel volume) of phenyl-Sepharose (GE Healthcare UK Ltd., Buckinghamshire, UK) with constant agitation for 4 h at 4 °C. In this procedure P-SPHK2 was bound to the gel with an approximate recovery of 60%. The gels were collected by centrifugation and subjected to SDS-PAGE followed by immunoblotting using anti-SPHK2 (Santa Cruz Biotechnology) or anti-P-SPHK2 antibody.

Immunofluorescence—Cells were grown on four-chambered slides (Nalge/Nunc) and transfected using Fugene-6 reagent according to the manufacturer’s instructions (Roche Applied
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Science). Subcellular localization studies using confocal microscopy were performed as described previously (29). Antibodies used were polyclonal anti-phosphoSPHK2 (see above; 1:1000) and polyclonal anti-SK2 (1:1000). The fluorescence of Alexa488 and Alexa594 was observed under a confocal laser-scanning microscope (LSM 510 META, Carl Zeiss, Jena, Germany), with excitation at 488 nm using a 505–530 nm band-pass barrier filter and with excitation at 543 nm using a 560 nm long-pass barrier filter, respectively.

Fluorescence Recovery after Photobleaching—The FRAP analysis was performed with the Zeiss LSM 510 META confocal laser-scanning microscope as described (30). Briefly, HeLa cells transfected with SPHK2-green fluorescent protein (GFP) were treated without or with 100 nm PMA for 30 min, and then each nuclear or cytoplasmic region of interest was photobleached by scanning for 8 s with an argon laser at the highest power. Recovery of fluorescence in the nuclei after nuclear photobleach (nuclear import) or decrease in fluorescence in the nuclei after cytoplasmic photobleach (nuclear export) was then analyzed by confocal fluorescence microscopy with low laser power at the indicated times after photobleaching. For all of the images, the noise levels were reduced by line scan averaging.

Real-time Quantitative Reverse Transcription-PCR—Total RNA was extracted from HeLa cells (2 × 10⁶ cells) using ISOGEN (Nippon gene, Toyama, Japan) according to the manufacturer’s instruction. cDNA synthesis and real-time quantitative PCR were carried out as described previously (19). The primer sequences (sense and antisense) were as follows: human PKD1, 5'-CCA GGA AGG CGA TCT TAT TGA A-3' and 5'-GCT GGA GCT CTG TAT GAA TGA ACA-3'; human PKD2, 5'-AGC AAC AAC GAC ACG CTG AGA-3' and 5'-GAT TTC TGA CAG CGG AAT TTC C-3'; human PKD3, 5'-GGG CAA GGG AAA GAT CAC AA-3' and 5'-TTC CTC CAT AAA CGA TGC CAA AC-3'; human glyceraldehyde-3-phosphate dehydrogenase, 5'-GCC ATC AAT GAC CCC TTC ATT-3' and 5'-TCT CGC TCC TGG AAG ATG G-3'. The expression of each mRNA was normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA expression.

RESULTS

Phorbol Ester Induces the Phosphorylation of SPHK2—Post-translational phosphorylation of proteins is an important modification regulating protein function, localization, and stability. To study whether SPHK2 can be phosphorylated, HeLa cells transiently expressing HA-SPHK2 were treated with a tumor promoter, phorbol 12-myristate 13-acetate (PMA), which is known to induce phosphorylation of various cellular proteins (31). Treatment of cells with PMA for 2 h led to strong phosphorylation of SPHK2 as assessed by the incorporation of [32P]inorganic phosphate for 18 h. Cells were treated without or with 100 nm PMA for 30 min. After cell lysis SPHK2 was immunoprecipitated with anti-HA antibody followed by immunoblot analysis with the same antibody. The immunoblotted sheets were also subjected to autoradiography (Auto.). The data presented are a typical representative of three independent experiments. B, HeLa cells transiently expressing HA-SPHK2 were treated without or with 100 nm PMA for 30 min. Cells were permeabilized and immunostained for confocal microscopic analysis using anti-SPHK2 antibody (red). Nuclei were stained with 2 μg/ml 4,6-diamino-2-phenylindole (DAPI) (blue) and a differential interference contrast (DIC) image of each area is also presented. Bars, 10 μm. The data presented are a typical representative of four independent experiments. C, from immunocytochemical data obtained in B, cells expressing SPHK2 were subdivided into three populations depending on SPHK2 staining pattern: cells with predominantly nuclear localization (C < N), cells with equal distribution between nucleus and cytoplasm (N = C), and cells with predominantly cytoplasmic localization (N > C). Five different fields were analyzed with a minimum of 100 cells per field. The data presented are a typical representative of four independent experiments.

Because protein modifications can affect the function and localization of proteins, and because SPHK2 was phosphorylated by PMA treatment, we studied the effect of PMA on the localization of SPHK2, which under basal conditions is localized predominantly in the nucleus of HeLa cells. We observed that, in HeLa cells expressing HA-SPHK2, when treated with PMA, under conditions where phosphorylation is strongly induced, a redistribution of SPHK2 from the nucleus to the cytoplasm occurred (Fig. 1, B and C).

PMA Induces the Nuclear Export of SPHK2—PMA-induced cytoplasmic accumulation of SPHK2 could be due to an increase in the nuclear export or an inhibition of nuclear import processes. To identify which of these mechanisms was important for the observed effects, the PMA-induced shuttling of SPHK2 was further characterized by FRAP analysis. HeLa cells transiently expressing SPHK2-GFP were analyzed for the dynamics of the fluorescent protein after photobleaching. When the fluorescent protein in the nucleus was photobleached, recovery of nuclear fluorescence was almost indistinguishable between control and PMA-treated cells (Fig. 2, A and B). On the other hand, when fluorescence in the cytoplasmic region was photobleached, the rate of decrease in fluorescence...
intensity in the nuclear region was markedly (4-fold) enhanced by PMA treatment. These results indicate that PMA mainly causes an increase in the nuclear export of SPHK2, rather than an inhibition of nuclear import which results in the accumulation of SPHK2 in the cytosol. Further, we found that when HEK293 cells expressing HA-SPHK2 were treated with leptomycin B it led to an accumulation of SPHK2 in the nucleus. Leptomycin B can bind covalently to a cysteine residue in the nuclear export receptor chromosomal region maintenance 1 (CRM1) (32) and interfere with the binding of both Ran and the cargo proteins (33, 34), thereby inhibiting CRM1-mediated nuclear export of cargo proteins. This indicates a CRM1-mediated nuclear export of SPHK2 (data not shown). Taken together, these results along with those of Fig. 1 suggest a strong correlation between protein phosphorylation and nuclear export of SPHK2.

Identification of NES in SPHK2—Proteins that are exported from nucleus in a CRM1-dependent manner are known to contain NES sequences, which consist of several leucine residues distributed with an uneven spacing (35, 36). As PMA-induced localization changes of SPHK2 were predominantly related to nuclear export, we sought to identify and characterize one or more NESs in the SPHK2 molecule. Close inspection of the SPHK2 sequence revealed at least one putative leucine-rich NES-like motif in the middle divergent region of the protein (Fig. 3A) along with several other potential NES-like sequences that were not investigated further during the present studies. It

FIGURE 2. Regulation of nucleo/cytoplasmic shuttling of SPHK2 through nuclear export as demonstrated by FRAP analysis. HeLa cells were transiently transfected with an expression vector encoding SPHK2-GFP. Two days after transfection living cells were treated without or with 100 nM PMA for 30 min and subjected to FRAP analysis using confocal laser-scanning microscopy. Either nuclear regions (Nuclear import) or cytoplasmic regions (Nuclear export) were photobleached. Subsequently, images were collected at the indicated time points. Representative images before and after (0 and 10 min) PMA treatment are shown (A). For the graphs the fluorescence recovery after photobleach at the nuclear region of interest was measured and is given as percent fluorescence recovery based on the initial value after photobleaching (the lowest fluorescence intensity in nuclear import and the highest fluorescence intensity in nuclear export at 0 s) (B). Data are means ± S.E. of three independent experiments carried out in triplicate.

FIGURE 3. Identification of NES in SPHK2. A, a schematic representation of NES sequence. Two conserved catalytic domains are shown in gray and black boxes. The NES is located in the middle divergent sequence. The asterisks indicate the leucine residues in NES sequence that were mutated to alanine residues. B, HEK293 cells were transiently transfected with a plasmid vector encoding HA-SPHK2 or HA-SPHK2L423A/L425A (NES mutant). Two days after transfection cells were fixed, permeabilized, and immunostained for confocal microscopic analysis using anti-SPHK2 antibody (red). Nuclei were stained with DAPI (blue). DIC image of each area is also shown. Bars, 10 µm. C, subcellular localization of SPHK2 obtained in panel B was quantified and classified as in Fig. 1. The data presented are a typical representative of three independent experiments.
is known that changing the hydrophobic leucine residues of many CRM1-dependent NES sequences to alanine abrogates the export function of the NES (35–37). To determine whether the NES-like sequence was functional in the wild-type protein, we constructed an alanine substitution mutant in the NES-like sequence (NES mutant: leucines 423 and 425 into alanine, L423A/L425A) and studied its subcellular distribution when expressed in HEK293 cells. HEK293 cells were chosen, because transiently expressed recombinant SPHK2 is localized mainly in the cytosol of these cells. When the NES mutant was expressed in HEK293 cells, the number of cells showing nuclear predominant localization of the mutant protein was mildly increased (2- to 3-fold) compared with cells expressing wild-type protein (Fig. 3, B and C), suggesting that the NES is functional under basal conditions. It should be noted that the expression level and in vitro enzymatic activity of the NES mutant were similar to that of the WT protein indicating the mutations did not lead to gross structural changes in the protein (data not shown).

Phosphorylation of SPHK2 at Serine Residue(s) within the NES and Its Nuclear Exit—Phosphorylation or dephosphorylation of nuclear shuttling proteins especially close to the NLS (38, 39) or NES (40–42) is known to regulate the shuttling of these proteins. Because the NES is working weakly under basal conditions (Fig. 3) and contains two potential phosphorylation sites just after arginine 418 within its sequence, we speculated that one or both of the serine residues (419 and 421) in the NES may be the target of PMA-induced phosphorylation that is important for the regulation of the nuclear shuttling of this protein. To demonstrate this we constructed an alanine substitution mutant SPHK2S419A/S421A in the NES sequence (phosphorylation-deficient mutant: serines 419 and 421 into alanine) (Fig. 4A) and compared its subcellular localization with wild-type SPHK2 after expression in HeLa cells. The mutation did not affect the enzymatic activity or the expression of the mutant SPHK2 (data not shown). As expected, in contrast to wild-type SPHK2 the phosphorylation-deficient mutant lost its ability to exit from the nucleus in response to PMA treatment (Fig. 4, B and C), suggesting that nucleo/cytoplasmic shuttling of SPHK2 may be regulated by phosphorylation of the serine(s) in the NES.

To further implicate the importance of phosphorylation of SPHK2 during its nuclear export, we generated an antibody that specifically recognizes the phosphorylated NES which has two serine residues at positions 419 and 421 that can potentially be phosphorylated. To show the specificity of this phosphorylated SPHK2 (P-SPHK2)-specific antibody SPHK2 was phosphorylated in a purified in vitro system by PKD and subjected to immunoblot analysis. P-SPHK2-specific antibody recognized the protein only when ATP was included in the assay (Fig. 5, A and B), validating the antibody specificity. P-SPHK2-specific antibody recognized the protein when HeLa cells transiently expressing SPHK2 were treated with PMA but not in untreated cells (Fig. 5C). Of note, anti-P-SPHK2 antibody did not recognize phosphorylation-deficient mutant irrespective of PMA treatment. In addition, this antibody recognized endogenous SPHK2 when HepG2 cells were treated with PMA (Fig. 5D). Next, phosphorylation of SPHK2 was assessed by immunocytochemical analysis using anti-P-SPHK2 antibody. This antibody could detect cytoplasmic SPHK2 in PMA-treated HeLa cells expressing SPHK2-GFP but not in untreated cells (Fig. 5E), implicating that PMA-induced phosphorylation of Ser-419/Ser-421 in the NES of SPHK2 facilitates the nuclear export of this enzyme. To show that phosphorylation of SPHK2 in its NES sequence and its subsequent nuclear export apply not only to overexpressed recombinant protein but the endogenous one too, subcellular localization of SPHK2 was studied in human hepatoma HepG2 cells. Using anti-SPHK2 antibody SPHK2 was stained mainly in the nucleus with a clear nucleo/cytoplasmic boundary. The amount of P-SPHK2, as evidenced by an increase in staining intensity, was significantly increased in the cytoplasm of PMA-treated cells as compared with that in untreated control cells. These results suggest a physiological relevance of NES phosphorylation of

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SPHK2 in the regulation of nucleo/cytoplasmic shuttling of this protein.

PKD-mediated Phosphorylation of SPHK2 at Serine Residue(s) within the NES—Next, a series of experiments was conducted to identify a protein kinase responsible for the phosphorylation of Ser-419/Ser-421 in the NES of SPHK2. From the sequence analysis the NES shares similarity with the consensus sequence (LXRXS) (Fig. 6A) for phosphorylation by PKD (43). To assess whether PKD was responsible for phosphorylation of SPHK2 under PMA stimulation, in vitro phosphorylation of the NES peptide by PKD was carried out. An affinity-purified constitutively active PKD3 efficiently phosphorylated the NES peptide. Phosphorylation of syntide-2, an authentic PKD substrate, was used as a control (Fig. 6B). SPHK2 phosphorylation by PKD was further assessed in an intact cell system. HeLa cells were transiently transfected both with SPHK2-GFP and either an empty vector or a plasmid encoding HA-tagged constitutively active PKD3 and analyzed by immuno- cytochemistry using anti-SPHK2 or anti-P-SPHK2 antibody. When SPHK2-GFP was expressed alone, it was distributed predominantly in the nucleus with weak cytoplasmic staining (Figs. 5B and 6C). In contrast, when SPHK2-GFP and constitutively active PKD3 were transiently co-expressed in HeLa cells, SPHK2 was localized mostly in the cytosol (Fig. 6C). Anti-P-SPHK2 antibody reacted with SPHK2 more strongly in the cytosol of cells expressing the active PKD than in cells transfected with an empty vector. When phosphorylation-deficient mutant SPHK2S419A/S421A-GFP was expressed, the mutant protein was not detected by anti-P-SPHK2 antibody irrespective of active PKD expression and remained in the nucleus. These results indicate that PKD has the ability to phosphorylate SPHK2 both in vitro and in vivo.

PKD as a Physiologically Relevant Protein Kinase for the Phosphorylation of SPHK2—It is important to determine whether PKD is a physiologically relevant protein kinase that phosphorylates SPHK2 for the regulation of nucleo/cytoplasmic shuttling under various conditions, including PMA stimulation. To address this issue phosphorylation

FIGURE 5. Detection of PMA-induced Ser-419/Ser-421 phosphorylation using a phospho-specific antibody. Specificity of anti-P-SPHK2 antibody was demonstrated by showing the reactivity of the antibody only with the phosphorylated form. Affinity-purified HA-SPHK2 and HA-PKD3 were incubated without or with 100 μM ATP in kinase buffer. Samples were subjected to SDS-PAGE followed by immunoblotting with anti-SPHK2, anti-P-SPHK2, or anti-HA antibody (A). Immunoblot results obtained in A were quantitated (B). C, HeLa cells were transiently transfected with expression vector encoding HA-SPHK2 or HA-SPHK2S419A/S421A. Two days after transfection cells were treated with 100 nM PMA for 30 min followed by immunoblot analysis using anti-P-SPHK2 antibody or anti-HA antibody. The data presented are a typical representative of five independent experiments. D, HepG2 cells were treated without or with 100 nM PMA for 1 h. After cell lysis SPHK2 was concentrated by phenyl-Sepharose and subjected to SDS-PAGE followed by immunoblot analysis with anti-SPHK2 or anti-P-SPHK2 antibody. E, HeLa cells transiently expressing SPHK2-GFP and HepG2 cells were treated without or with 100 nM PMA for 30 min. Cells were permeabilized and immunostained for confocal microscopic analysis using anti-SPHK2 or anti-P-SPHK2 antibody. For HeLa cells SPHK2 is green and P-SPHK2 is red; for HepG2 cells SPHK2 and P-SPHK2 are red and DIC images are shown. Nuclei were stained with DAPI (blue). Bars, 10 μm. The data presented are a typical representative of three independent experiments.
had almost no effect, whereas PKD3- and PKD2-siRNA treatment caused a strong (70%) and a moderate (50%) inhibition of PMA-induced phosphorylation of SPHK2, respectively, consistent with the level of the isozyme expression (Fig. 7A). Combination of PKD2- and PKD3-siRNA treatment caused a stronger inhibition (90%). Silencing of all the three PKD subtypes by specific siRNAs simultaneously resulted in an almost complete loss of PMA-induced phosphorylation of SPHK2, suggesting a redundant function for PKD subtypes in the phosphorylation of SPHK2. Indeed, expression of a constitutively active PKD1 in HeLa cells resulted in potent phosphorylation of SPHK2 and its exit from the nucleus under basal conditions (data not shown). Consistent with our hypothesis that SPHK2 undergoes PKD-mediated phosphorylation in response to PMA treatment and exits from the nucleus, immunocytochemical analysis of HeLa cells where endogenous PKD was down-regulated by all three PKD subtype-siRNA-treated cells PMA-induced phosphorylation of SPHK2 and its subsequent exit from the nucleus were abrogated in PKD-knockdown cells (Fig. 7C and D). Combination of PKD2- and PKD3-siRNA treatment gave similar results (see supplemental Fig. S1). Expression of an siRNA-resistant PKD3 in the PKD-knockdown cells almost completely restored the PMA-induced phosphorylation of serine residue(s) in the NES of SPHK2 and its subsequent exit from the nucleus (Fig. 7C, compare PKD3-expressing cells (arrows) with PKD3-non-expressing cells (arrowheads)).

Next, to assess the involvement of PKD in SPHK2 phosphorylation under physiological conditions we examined whether subcellular distribution of SPHK2 in HEK293 cells was influenced by down-regulating PKD expression, because this enzyme was localized mainly in the cytosol in these cells under basal conditions (Fig. 3). As expected, down-regulation of PKD expression by PKD-siRNA treatment resulted in an increase in the cell population showing localization of SPHK2 predominantly in the nucleus (Fig. 7E). Again, this change in subcellular distribution of SPHK2 caused by PKD down-regulation coincided with decreased phosphorylation levels assessed by anti-P-SPHK2 antibody (Fig. 7F). These results strongly indicate that PKD is a physiologically relevant protein kinase for the phosphorylation of serine residue(s) in the NES of SPHK2, which is important for its nuclear export both under basal as well as agonist-stimulated conditions.

**DISCUSSION**

We have identified and characterized for the first time the NES in SPHK2 critically involved in its nuclear export. The NES is weakly functional under basal conditions (Fig. 3, B and C). Upon stimulation with PMA, SPHK2 undergoes phosphorylation of serine residue(s) in the NES and exits from the nucleus.
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A

PKD mRNA expression (% of GAPDH mRNA) by siRNA

B

P-SPHK2

SPHK2

PMA

Control siRNA

PKD1-siRNA

PKD2-siRNA

PKD3-siRNA

PKD1 + PKD2 + PKD3 siRNA

C

Control siRNA, PMA

PKD-siRNA, PMA

PKD-siRNA + PKD3, PMA

D

Cell populations expressing SPHK2 (%)

E

Cell populations with predominantly nuclear localization of HA-SPHK2 (%)

F

P-SPHK2

SPHK2

Control siRNA

PKD-siRNA
Our studies also revealed for the first time that SPHK2 is post-translationally modified by a novel mechanism involving PKD. Studies using siRNA-mediated silencing of PKD expression (Fig. 7, C and D) as well as phosphorylation of SPHK2 by PKD3 using in vitro (Fig. 6B) and in vivo (Fig. 6C) systems demonstrated that PKD is a physiologically relevant protein kinase for the phosphorylation of serine residue(s) in the NES of SPHK2 in response to various stimuli, including PMA. A phosphorylation-deficient mutant involving serine residues 419 and 421 (SPHK2S419A/S421A) was inert to both phosphorylation and nuclear exit (Figs. 4B, 4C, and 6C). A phosphorylation-mimicking mutation in the NES, changing both serine residues to glutamic acid (SPHK2S419E/S421E), was unable to enhance the NES activity (data not shown). On the other hand, a phosphorylation-deficient mutant of a single serine (serine 419 to alanine SPHK2S419A) was exported from the nucleus in response to PMA just like the wild-type protein (data not shown). However, a phosphorylation-deficient mutation at a single serine 421 (SPHK2S421A) showed clear inhibition of the NES activity (data not shown) but not complete nuclear retention as in the double mutant (SPHK2S419A/S421A) (Fig. 4, B and C). For these reasons SPHK2S419A/S421A was used as a phosphorylation-deficient mutant during the present studies.

In addition to the NES sequence (amino acid residues 416–425) investigated in the present studies we identified several other NES-like sequences in SPHK2. One of them was located in the N-terminal conserved region (LDDLINC-SLL: amino acid residues 298–308). This putative NES also may be active under basal conditions (see supplemental Fig. S2). Interestingly, a mutant version of this putative NES-like sequence (leucines 306 and 308 into alanine, SPHK2L306A/L308A) lost its NES activity, but SPHK2 was heavily phosphorylated at serine residue(s) in the NES (amino acid residues 416–425) within the nucleus after PMA stimulation (see supplemental Fig. S3), suggesting that phosphorylation of SPHK2 may occur inside the nucleus. This notion was further strengthened by the observation that SPHK2 was heavily phosphorylated at Ser-419/Ser-421 of the NES in isolated nuclei obtained from PMA-treated HeLa cells transiently expressing the putative NES mutant SPHK2L306A/L308A (see supplemental Fig. S4). In addition, it has been reported that PKD translocates into nucleus upon stimulation of cells by PMA (44) where majority of SPHK2 is localized under basal conditions (Figs. 1, 5, and 6). Taken together, it may be reasonable to assume that phosphorylation of SPHK2 by PKD may occur inside the nucleus, although PKD has no interaction with SPHK2 as assessed by pull-down assay (data not shown).

We have previously reported that subcellular distribution of SPHK2 differs depending on cell type and cellular growth conditions. Upon expression, recombinant SPHK2 was localized mainly in the nucleus in HeLa cells, whereas the enzyme was distributed predominately in the cytoplasm of COS7 and HEK293 cells (18). Based on our hypothesis that SPHK2 undergoes phosphorylation and exit from the nucleus to cytoplasm, SPHK2 expressed in HEK293 cells should be phosphorylated more highly under basal conditions than that expressed in HeLa cells. In fact, SPHK2 expressed in HEK293 cells was localized mainly in the cytoplasm (Fig. 3) and was more strongly phosphorylated than that expressed in HeLa cells as determined by immunoblot analysis using anti-P-SPHK2 antibody (data not shown). Importantly, down-regulation of PKDs through RNA interference resulted in the accumulation of SPHK2 in the nucleus under basal conditions (Fig. 7E) with a concomitant decrease in the phosphorylation within the NES (Fig. 7F), indicating the physiological importance of PKD in this phosphorylation of SPHK2 and its subsequent export from the nucleus both under basal as well as agonist-stimulated conditions. We have previously shown that SPHK2 accumulates in the nucleus under stressful conditions such as high confluence or serum starvation, resulting in apoptosis or the inhibition of cell proliferation (18, 19). PKD may transmit such stressful signals by phosphorylating SPHK2. Mechanism underlying PKD activation under such conditions remains to be clarified.

The other isoform, SPHK1, is a cytosolic protein with an apparent molecular mass of 45 kDa. A functional NES for SPHK1 has not been identified yet. A protein of this molecular size may passively enter the nucleus. Recently, two functional NESs in SPHK1 have been identified (45). These NESs may be acting constitutively under basal conditions. Amino acid sequences around the phosphorylation sites of the NES in SPHK2 are highly conserved from rat to human, suggesting that the mechanism underlying the nucleo/cytoplasmic shuttling of SPHK2 may be common to mammalian species and be implicated in the regulation of vital cell functions. Based on sequence analysis SPHK1 has no consensus phosphorylation sequence for PKD. Even though SPHK1 and SPHK2 share similar catalytic properties, these two isoforms are differently regulated in topological distribution, suggesting distinct physiological functions. The PKD enzymes have recently been implicated in a variety of cellular functions, including Golgi organization and...

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FIGURE 7. Demonstration of PKD as a physiologically relevant protein kinase for Ser-419/Ser-421 phosphorylation of SPHK2. A, HeLa cells were transfected with control, PKD1-, PKD2-, PKD3-, or all three PKD-siRNAs and cultured for 72 h. Total RNA was isolated from the cells and reverse transcribed. The cDNAs were used for real-time quantitative PCR with primers specific for each PKD isoform and for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase. Values of PKD mRNA amounts were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression and are the mean \( \pm S.E \) from three independent experiments done in groups of five. B, HeLa cells were transfected with both HA-SPHK2 and various combinations of PKD isoform-specific siRNAs and cultured for 72 h. Cells were treated without or with 100 nM PMA for 30 min followed by immunoblot analysis using anti-HA or anti-P-SPHK2 antibody. C and D, HeLa cells were transfected with SPHK2-GFP and either empty vector or expression vector encoding siRNA-resistant PKD3 together with control or all three PKD-siRNAs and cultured for 72 h. Cells were treated without or with 100 nM PMA for 30 min, permeabilized, and immunostained for confocal microscopy using anti-SPHK, anti-P-SPHK2, and anti-HA antibodies (C, Bars, 10 \( \mu \)m. Subcellular localization of SPHK2 obtained in panel C was quantified and classified as in Fig. 1 (D). E and F, HEK293 cells were transfected both with HA-SPHK2 and either control or all three PKD-siRNAs and cultured for 72 h. Cells were fixed, permeabilized, and immunostained for confocal microscopic analysis using anti-SPHK2 antibody. E, subcellular distribution of SPHK2 was determined as in Fig. 1. F, aliquots of the samples were also subjected to SDS-PAGE followed by immunoblot analysis with anti-SPHK2 and anti-P-SPHK2 antibodies.
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plasma membrane-directed transport, metastasis, immune responses, and cell proliferation (44). Based on the present data the activation of PKD results in SPHK2 phosphorylation, which facilitates its nuclear export and may modulate functions such as cell proliferation and survival. The role of PKD needs to be re-evaluated in terms of cellular functions mediated by SPHK2.

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