δ-Catenin/NPRAP (neural plakophilin-related armadillo repeat protein) interacts with and activates sphingosine kinase 1

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Sphingosine kinase (SPHK) is a key enzyme catalysing the formation of sphingosine 1-phosphate (SPP), a lipid messenger that is implicated in the regulation of a wide variety of important cellular events acting through intracellular, as well as extracellular, mechanisms. However, the molecular mechanism of intracellular actions of SPP remains unclear. Here, we have identified δ-catenin/NPRAP (neural plakophilin-related armadillo repeat protein) as a potential binding partner for SPHK1 by yeast two-hybrid screening. From co-immunoprecipitation analyses, the C-terminal portion of δ-catenin/NPRAP containing the seventh to tenth armadillo repeats was found to be required for interaction with SPHK1. Endogenous δ-catenin/NPRAP was co-localized with endogenous SPHK1 and transfected δ-catenin/NPRAP was co-localized with transfected SPHK1 in dissociated rat hippocampal neurons. MDCK (Madin–Darby canine kidney) cells stably expressing δ-catenin/NPRAP contained elevated levels of intracellular SPP. In a purified system δ-catenin/NPRAP stimulated SPHK1 in a dose-dependent manner. Furthermore, δ-catenin/NPRAP-induced increased cell motility in MDCK cells was completely inhibited by dimethylsphingosine, a specific inhibitor of SPHK1. These results strongly suggest that at least some of δ-catenin/NPRAP functions, including increased cell motility, are mediated by an SPHK–SPP signalling pathway.

Key words: δ-catenin, cell motility, dimethylsphingosine, sphingosine kinase, sphingosine 1-phosphate.

INTRODUCTION

Sphingosine 1-phosphate (SPP), a sphingolipid metabolite, has recently received considerable attention as a novel signalling lipid that acts in mammalian cells as an intracellular second messenger, as well as a ligand for cell-surface receptors [1,2]. Notably, some of the diverse signalling roles attributed to elevated cellular SPP levels include prevention of ceramide-induced apoptosis [3,4], mobilization of intracellular calcium [5], stimulation of DNA-binding activity of the transcription factor activator protein-1 [6], activation of mitogen-activated protein kinase pathways [7,8], activation of phospholipase D [9] and stimulation of adhesion molecule expression [8].

Cellular levels of SPP are largely controlled by its formation from sphingosine by the activity of sphingosine kinase (SPHK), and to a lesser extent by its degradation by SPP lyase [10] and SPP phosphatase [11,12] activities. SPHK is activated by a variety of stimuli, including platelet-derived growth factor, epidermal growth factor, nerve growth factor, vitamin D_3, tumour necrosis factor-α and cross-linking of FcεRI and FcγRI (reviewed in [13,14]). To date, two isotypes of SPHK, SPHK1 and SPHK2, have been cloned and characterized that have both conserved catalytic domains and divergent sequences, especially in the N-terminus and central region of SPHK2 [15,16], implying distinct physiological functions [17–19]. Despite the importance of SPHK, little is known regarding the molecular mechanisms of activation and intracellular site of action of the enzymes.

In order to identify proteins that may interact with SPHK, we screened a rat brain cDNA library using a yeast two-hybrid procedure with a mouse SPHK1 construct as bait. We isolated δ-catenin/NPRAP (neural plakophilin-related armadillo repeat protein) as a potential binding partner for SPHK1. δ-Catenin/NPRAP is a member of the $p120^{ctn}$ subfamily enriched in the brain. This subfamily is defined as proteins with 10 armadillo repeats in a characteristic spacing and often with quite diverse N- and C-terminal sequences flanking the repeats that can potentially participate in protein–protein interactions [20–23] and protein phosphorylations [24]. These features indicate that δ-catenin/NPRAP may be a scaffolding/adaptor protein that can couple or modulate signal-transduction events.

We have obtained evidence for an association of SPHK1 with δ-catenin/NPRAP *in vivo*, as well as *in vitro*. We also provide evidence to indicate that some of the physiological functions induced by δ-catenin/NPRAP may be mediated by the SPHK1–SPP pathway.

EXPERIMENTAL

Yeast two-hybrid screening

Full-length mouse SPHK1a cDNA (GenBank® accession number, AF068748) was cloned into pGBKT7 (ClonTech, Palo Alto, CA, U.S.A.) 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). DNA from positive clones was prepared from yeast and transformed into competent DH5α cells (Takara, Otsu, Japan) according to standard protocols.

cDNA cloning and mammalian expression vectors

The human δ-catenin/NPRAP (GenBank® accession number, NM_001332) cDNA was amplified from a total human brain cDNA, which had been reverse transcribed from fetal human brain mRNA (Invitrogen, Carlsbad, CA, U.S.A.) by PCR using KOD-PLUS polymerase (Toyobo, Tokyo, Japan) with the primers 5′-GG GGT ACC ATG GAC TAC AAG GAC GAT GAT GAC

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; DMS, dimethylsphingosine; FBS, fetal bovine serum; HA, haemagglutinin; HGF, hepatocyte growth factor; MDCK, Madin–Darby canine kidney; NPRAP, neural plakophilin-related armadillo repeat protein; SPHK, sphingosine kinase; SPP, sphingosine 1-phosphate.

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AAG ATG TTT GCG AGG AAG CCG CC-3′ (sense) and 5′-GC TCT AGA TCA CAC CCA GGA GTC GGG GGA G-3′ (antisense), to make N-terminally FLAG-tagged construct. For the δ-catenin/NPRAP(ΔN1017) construct (deletion of amino acids 1–1017 at the N-terminus), the primers used were 5′-CGG GGT ACC CAG TAC CGA GAT CTG AGG AGT CTC-3′ (sense) and full-length δ-catenin/NPRAP (antisense). For the δ-catenin/NPRAP(ΔN832) construct (lacking amino acids 1–832 at the N-terminus), the primers used were 5′-CGG GGT ACC GAA CCA CCA AAA GGG ATC C-3′ (sense) and full-length δ-catenin/NPRAP (antisense). For the δ-catenin/NPRAP(ΔC209) construct (deletion of amino acids 1017–1225 at the C-terminus), the primers used were full-length δ-catenin/NPRAP (sense) and 5′-GC TCT AGA TCA CCA CAT GCT GTT GAG GAC CTG-3′ (antisense). These products were then inserted between *Kpn*I and *Xba*I sites of pCMV5. pCMV5-influenza haemagglutinin (HA)-mouse (m)SPHK1 was prepared as previously described [19].

The FLAG–δ-catenin/NPRAP, FLAG–δ-catenin/NPRAP-(ΔN1017) or HA–SPHK1 cDNA was also subcloned into pIRESneo2 plasmid (ClonTech) for use in stable transfection. MDCK (Madin–Darby canine kidney) cells were transfected with these plasmids by electroporation. Drug-resistant clones were isolated in the presence of 1 mg/ml Geneticin (Invitrogen).

Cell culture and immunocytochemistry

COS7 and HEK-293 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % FBS (fetal bovine serum), 100 units/ml penicillin and 100 μg/ml streptomycin in 5 % CO_2 atmosphere/95 % air at 37 °C with 100 % humidity. MDCK cells stably expressing either FLAG–δ-catenin/NPRAP or HA–SPHK1 were cultured similarly except that, instead of penicillin and streptomycin, 1 mg/ml geneticin was included in the medium.

Primary hippocampal neurons were cultured as described [25] with some modifications. Briefly, 18-day pregnant Wistar rats were killed and the embryos were removed. Hippocampi were dissected, and dissociated by 0.05 % papain (Nacalai Tesque, Kyoto, Japan) treatment. The dissociated cells were plated on to coverslips coated with poly-L-lysine (Sigma) and laminin (Asahi Techno Glass, Tokyo, Japan) and incubated in Neurobasal medium (Invitrogen) supplemented with 10 % FBS. After 3 h incubation the medium was changed to Neurobasal medium containing B27 supplement (Invitrogen), 1 mM glutamine, 2.5 μM cytosine β-D-arabinofuranoside (Sigma), 100 units/ml penicillin and 100 μg/ml streptomycin. Hippocampal neurons grown in culture for 1 day were transfected with both FLAG-δ-catenin/NPRAP- and HA-mSPHK1-containing vectors using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions and cultured for 2 days. Neurons were fixed in 4 % paraformaldehyde for 20 min, permeabilized with 0.2 % Triton X-100 for 10 min, and blocked by 1 % BSA in PBS. Non-transfected neurons were double-stained with a mouse monoclonal anti-δ-catenin/NPRAP antibody (BD Biosciences, San Jose, CA, U.S.A.) and a goat polyclonal anti-SPHK1 antibody (Abcam, Cambridge, U.K.) and visualized with Alexa 594-conjugated rabbit anti-(mouse IgG) and Alexa 488-conjugated donkey anti-(goat IgG) antibodies. Transfected neurons were double-stained with a mouse monoclonal anti-FLAG antibody M2 (Sigma) and a rat monoclonal anti-HA antibody (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.) and visualized with Alexa 594-conjugated rabbit anti-(mouse IgG) and Alexa 488-conjugated rabbit anti-(rat IgG). Subcellular localization studies using confocal microscopy were performed as described previously [26].

Immunoprecipitation

COS7 cells transiently expressing various FLAG–δ-catenin/NPRAP mutants along with HA–mSPHK1 were lysed in cold lysis buffer [20 mM Tris/HCl, pH 7.4, 100 mM NaCl, 1 % (w/v) Brij 97 (Sigma) containing 'complete' protease inhibitors (Roche Molecular Biochemicals)]. Immunoprecipitation and immunoblot analyses were carried out as described previously [27] using a mouse monoclonal anti-FLAG antibody M2 and a rat monoclonal anti-HA antibody. To estimate the expression of endogenous SPHK1 in Figure 3, immunoblot analyses were conducted using a rabbit polyclonal anti-SPHK1 antibody kindly provided by Dr Yasuyuki Igarashi (Department of Biomembrane and Biofunctional Chemistry, Graduate School of Pharmaceutical Sciences, Hokkaido University, Japan).

In vitro SPHK assay

HA–mSPHK1, FLAG–δ-catenin/NPRAP and FLAG–δ-catenin-(ΔN1017) were purified by using either anti-HA antibody beads (Roche Molecular Biochemicals) or anti-FLAG antibody M2 beads (Sigma) and finally eluted with HA or FLAG peptide (Sigma) respectively, according to the manufacturer's protocol. In some experiments post-nuclear supernatants from MDCK cells were used as enzyme sources. SPHK activity was determined in the reaction mixture (50 μl) containing 10 μM sphingosine, 1 mM [γ-^{32}P]ATP (8 Ci/mol), and 10 mM $MgCl_2$ essentially as described previously [28]. [^{32}P]SPP was separated by TLC on Silica Gel 60 (Merck) with 1-butanol/acetic acid/water (3:1:1, by vol.) and quantified using a Fujix Bio-Imaging Analyzer BAS 2000 (Fuji Photo Film).

Intracellular SPP level

MDCK cells stably expressing FLAG–δ-catenin/NPRAP, FLAG–δ-catenin/NPRAP(ΔN1017) or HA–SPHK1 grown in 6-cm diameter dishes were metabolically labelled with [^{32}P]orthophosphate (40 μCi/dish) for 24 h in phosphate-free DMEM. Sphingosine (500 μM; in 20 mM Hepes/NaOH, pH 7.4, and 1 % BSA) was added to the medium to adjust the final concentration of sphingosine to 5 μM. In some experiments various concentrations of dimethylsphingosine (DMS) (Sigma) or F12509A (kindly donated by Dr T. Kohama, Sankyo, Tokyo) were added together with sphingosine as indicated in the figure legends. After 1 h incubation with added sphingosine and/or inhibitors, the medium was discarded and 400 μl of methanol/1 M NaCl/5 M NaOH (100:100:3, by vol.) was added to the dishes. Cells were scraped on ice, removed to an Eppendorf tube, and 200 μl of chloroform was added. After mixing and centrifugation at 14 000 ***g*** for 5 min, the upper aqueous phase was transferred into a clean Eppendorf tube. After adding 20 μl of 1 M HCl and 400 μl of chloroform/methanol/HCl (100:200:1, by vol.) and mixing, samples were allowed to stand for 5 min at 20 °C. Then, 120 μl of chloroform and 120 μl of 2 M KCl were added, mixed and allowed to stand for another 5 min at 20 °C. After centrifugation at 14 000 ***g*** for 5 min, the lower organic phase was collected, dried under vacuum and dissolved in 20 μl of chloroform/methanol (1:1, v/v). [^{32}P]SPP was separated by TLC on Silica Gel 60 with chloroform/methanol/water (65:35:8, by vol.) and quantified as described above.

Cell migration assay

For cell migration analyses, MDCK cells stably expressing FLAG–δ-catenin/NPRAP or HA–mSPHK1 were collected by trypsinization, washed once in DMEM with 10 % FBS, and washed twice in serum-free DMEM. The cells (3.5×10^4) were

then resuspended in 70 μl of DMEM containing 0.1 % BSA and various concentrations of DMS, as indicated in the figure legends, and added into the upper chambers of the 96-well chemotaxis chamber (Neuro Probe, Cabin John, MD, U.S.A.). The lower chambers were filled with DMEM with 0.1 % BSA and the same concentrations of DMS as in the corresponding upper chambers. To investigate the effect of HGF (hepatocyte growth factor) (Sigma) on cell motility, 10 ng/ml HGF was added into the lower chambers. Cells in the upper chamber were allowed to migrate through a porous (8-μm pore size) membrane pre-coated with 0.01 % collagen type 1 (IFP, Yamagata, Japan) towards the lower chamber for 8 h at 37 °C. Cells on the upper surface of each filter were wiped off, whereas cells that had traversed to the bottom surface of the filter were fixed and stained using Diff-Quick (International Reagent, Hyogo, Japan) and counted under a microscope. Mean values from 10 randomly selected unit areas were calculated for each of the triplicate chambers. The results are presented as a percentage of the control values without DMS.

RESULTS

Identification of δ-catenin/NPRAP as an SPHK1-interacting protein

We performed yeast two-hybrid screening of a rat brain cDNA library using the full-length mouse SPHK1 cDNA as bait. Two clones were obtained from screening 1×10^6 clones. DNA sequence and database analysis revealed that these positive clones contained a partial sequence of mouse δ-catenin/NPRAP corresponding to amino acid position 833 to the terminal 1225. A cDNA encompassing the entire coding region of human δ-catenin/NPRAP was then obtained using a human brain cDNA library.

SPHK1 interacts with the C-terminal region of armadillo repeats of δ-catenin/NPRAP

To verify the interaction between SPHK1 and δ-catenin/NPRAP obtained from the results of yeast two-hybrid screening, we examined the *in vitro* interaction of SPHK1 with δ-catenin/NPRAP. COS7 cells were co-transfected with various FLAG–δ-catenin/NPRAP and HA–SPHK1 constructs, and cell lysates were immunoprecipitated with anti-FLAG antibody followed by immnoblotting with anti-HA antibody. SPHK1 specifically interacted with full-length δ-catenin/NPRAP (Figure 1B, lane 4), whereas the anti-FLAG antibody did not co-immunoprecipitate SPHK1 in the absence of full-length δ-catenin/NPRAP (Figure 1B, lane 2). A deletion mutant ΔN832, which lacks amino acids 1–832 at the N-terminus (Figure 1A), also specifically interacted with SPHK1 (Figure 1B, lane 6), confirming the yeast two-hybrid screening results. On the other hand, another mutant ΔN1017 (deletion of amino acids 1–1017 at the N-terminus), which does not contain the armadillo repeats, failed to interact with SPHK1 (Figure 1B, lane 8), suggesting the importance of the seventh to tenth armadillo repeats for SPHK1 binding. Indeed, a mutant ΔC209 (deletion of amino acids 1017–1225 at the C-terminus), which contains all 10 armadillo repeats, bound to SPHK1 (Figure 1B, lane 10). We were unable to show the binding of the seventh to the tenth armadillo repeat portion of δ-catenin/NPRAP to SPHK1, because of low protein expression from the plasmid DNA encoding this portion of δ-catenin/NPRAP for unknown reasons.

δ-Catenin/NPRAP is co-localized with SPHK1 in dissociated primary hippocampal neurons

As δ-catenin/NPRAP is a brain-specific member of p120 catenin subfamily [29,30], we examined cellular localization of both

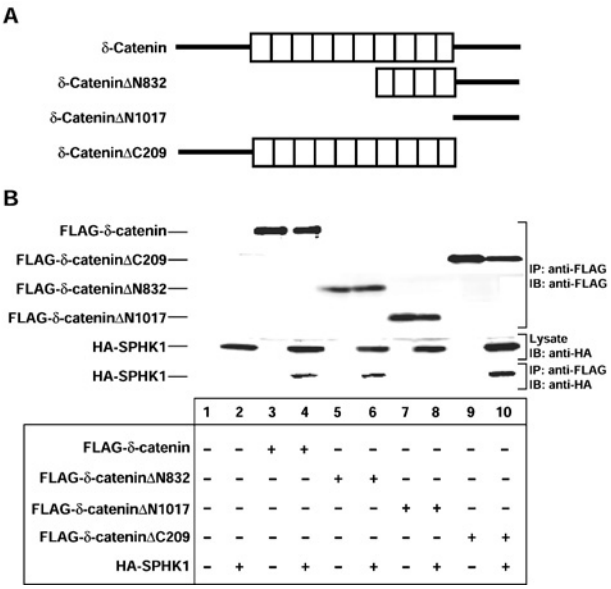

Figure 1 Interaction of δ-catenin/NPRAP with SPHK1

(**A**) A schematic representation of δ-catenin/NPRAP constructs that were used in the interaction studies. The armadillo repeats are shown as boxes. (**B**) Interaction between various δ-catenin/NPRAP constructs and SPHK1. COS7 cells were transiently transfected with various combinations of FLAG–δ-catenin/NPRAP constructs and HA–SPHK1 as indicated. Total amounts of plasmid DNA used for each transfection were adjusted to 1 μg by adding empty vector DNA. After cell lysis, FLAG-tagged proteins were immunoprecipitated by anti-FLAG antibody followed by immunoblotting with the anti-HA or anti-FLAG antibodies as indicated. The data presented are respresentative of five independent experiments. δ-catenin, δ-catenin/NPRAP.

δ-catenin/NPRAP and SPHK1 using endogenous proteins, as well as an exogenously introduced recombinant expression system, in primary rat hippocampal neurons cultured *in vitro* for 3 days. After transient transfection of hippocampal neurons with both FLAG–δ-catenin/NPRAP- and HA–mSPHK1-containing vectors, cells were immunostained and analysed by confocal microscopy. FLAG–δ-catenin/NPRAP was localized throughout the cell body and along the neuronal processes in small punctate structures (Figure 2A). HA–mSPHK1 staining showed a distribution similar to δ-catenin/NPRAP (Figure 2B) and was co-localized well, especially in the neuronal processes (Figure 2C, arrows). The staining pattern of endogenous δ-catenin/NPRAP and SPHK1 was quite similar to that of exogenously expressed proteins. δ-Catenin/NPRAP was localized both in the cell body and along the neuronal processes (Figure 2E). δ-Catenin/NPRAP was also concentrated in the growth cone (Figure 2E, asterisk). This pattern of localization is consistent with previous observations [24]. SPHK1 was also distributed in the periphery of cell body and punctate structures along the neuronal processes, showing good co-localization with δ-catenin/NPRAP in multiple punctate structures in the cell periphery and the neuronal processes (Figure 2G, arrows).

Expression of δ-catenin/NPRAP results in the accumulation of SPP in MDCK cells

It has been reported that over-expression of δ-catenin/NPRAP in MDCK cells results in an increase in cell motility [20]. It has also been shown that SPHK1 over-expression enhances cell motility [2,31]. The association of δ-catenin/NPRAP with SPHK1 obtained from yeast two-hybrid screening and co-immunoprecipitation assay *in vitro* (Figure 1), as well as immunofluorescence studies (Figure 2), prompted us to test whether increased cell

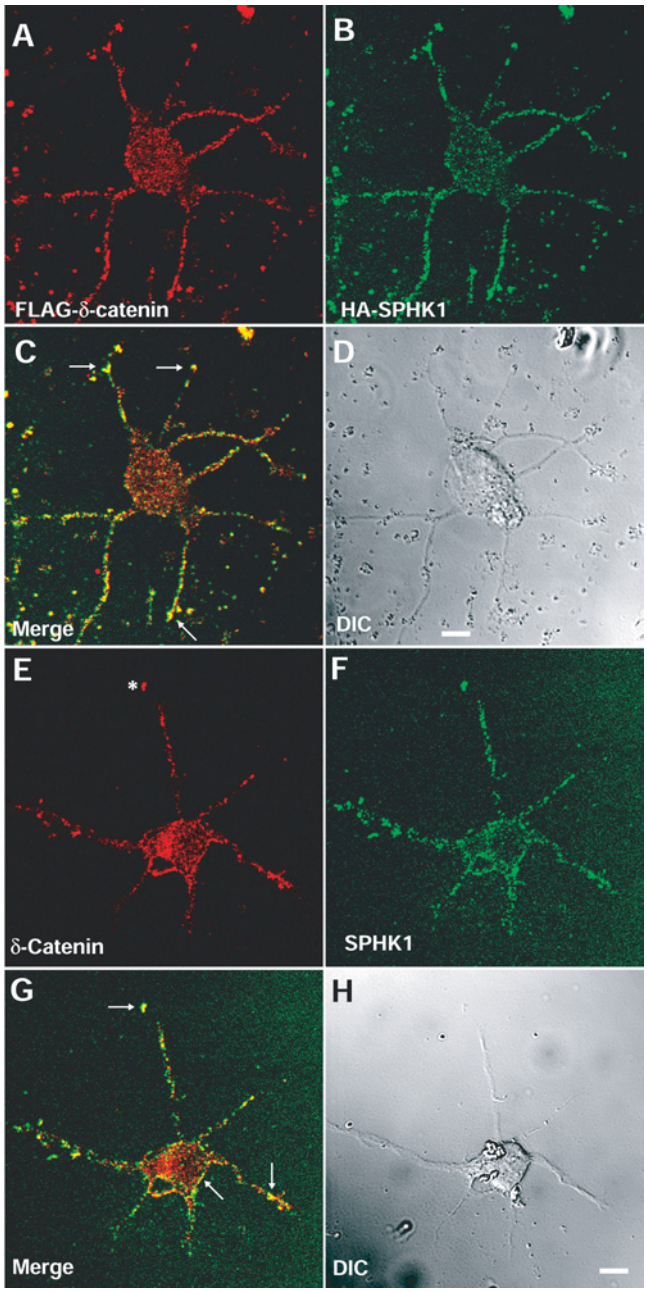

Figure 2 Co-localization of δ-catenin/NPRAP with SPHK1 in primary embryonic hippocampal neurons

Dissociated rat hippocampal neurons were transiently co-transfected with FLAG–δ-catenin/NPRAP and HA–SPHK1. Transfected (**A**–**D**) or non-transfected (**E**–**H**) neurons were doubly immunostained with anti-FLAG antibody (red) and anti-HA antibody (green) or anti-δ-catenin/NPRAP antibody (red) and anti-SPHK1 antibody (green), respectively. Yellow areas in the merged images (**C** and **G**) indicate co-localization of these proteins. Differential interference contrast images (**D** and **H**) are also shown. Scale bars, 10 μm.

motility caused by δ-catenin/NPRAP over-expression was mediated by the SPHK1–SPP pathway. To address this issue we measured the intracellular SPP content in MDCK cells stably expressing FLAG–δ-catenin/NPRAP. Over-expression of δ-catenin/NPRAP in MDCK cells resulted in about 3–4-fold increase in intracellular SPP content compared with MDCK cells transfected with empty vector (Figure 3A). A similar increase in SPP content was observed in MDCK cells over-expressing SPHK1. As expected, the expression of the deletion mutant δ-catenin (ΔN1017), which is devoid of the ability to interact with SPHK1 (Figure 1), resulted in almost negligible accumulation of SPP. To determine whether the increased accumulation of SPP observed in δ-catenin/NPRAP-expressing MDCK cells was due to increased production of SPP by SPHK or decreased degradation of SPP by SPP lyase or SPP phosphatase, the effect of specific SPHK inhibitors was tested. DMS (10 μM) almost completely inhibited the δ-catenin/NPRAP-induced SPP accumulation (Figure 3B). DMS at concentrations used in the present study is known to specifically inhibit SPHK activity, but not inhibit protein kinase C [32]. Another SPHK inhibitor, F12509A [33], also inhibited SPP accumulation in a dose-dependent manner, suggesting that δ-catenin/NPRAP expression enhances SPP accumulation by activating SPHK. To demonstrate an elevated SPHK activity in δ-catenin/NPRAP-expressing MDCK cells more clearly, SPHK activity of cell lysates prepared from MDCK cells stably transfected with either δ-catenin/NPRAP or empty vector was measured. As shown in Figure 3(C), the initial velocity of SPHK activity in δ-catenin/NPRAP-expressing MDCK cell lysates was 2–3 times higher than that in control lysates. The enhanced SPHK activity in δ-catenin/NPRAP-expressing MDCK cells may be due to the activation of SPHK1, but not due to the increased enzyme amount, as judged by the observation that the level of SPHK1 expression in MDCK cells stably expressing δ-catenin/NPRAP was almost unchanged when compared with that of empty vector-transfected MDCK cells (Figure 3D).

δ-Catenin/NPRAP activates SPHK1 *in vitro*

To elucidate the molecular mechanism of δ-catenin/NPRAP-induced accumulation of intracellular SPP in MDCK cells (Figure 3), the effect of δ-catenin/NPRAP on SPHK1 activity was studied using an *in vitro* assay system. When affinity-purified δ-catenin/NPRAP was added to SPHK assay mixture, it activated SPHK1 in a dose-dependent manner (Figure 4). Maximal activation (1.8-fold) was obtained with 50 ng of δ-catenin/NPRAP. Higher amounts of δ-catenin/NPRAP caused a slight inhibition of the enzyme. The deletion mutant δ-catenin(ΔN1017) had no effect on the enzymic activity.

Increased cell motility induced by δ-catenin/NPRAP over-expression is mediated by the SPHK1–SPP pathway in MDCK cells

To show the causal relationship between the cellular SPP content induced by δ-catenin/NPRAP over-expression in MDCK cells and the increase in cell motility more clearly, the effect of SPHK1 inhibitor DMS on cell motility was tested. When cell motility was measured using a two-chamber system, DMS dose-dependently inhibited δ-catenin/NPRAP-induced enhancement of cell migration in MDCK cells. SPHK1-over-expressing cells showed a similar extent of inhibition in response to various DMS doses (Figure 5). On the other hand, DMS had almost no effect on enhanced cell scattering upon stimulation by HGF (Figure 5), suggesting that the cellular signalling pathway induced by δ-catenin/NPRAP over-expression utilizes SPHK–SPP and is distinct from that stimulated by HGF.

DISCUSSION

Although the importance of intracellular actions of SPP is well recognized [34], the mechanism of action of SPP is poorly understood. For better understanding of intracellular actions of SPP, several approaches, including identification of SPHK-interacting molecules by yeast two-hybrid screening, were undertaken.

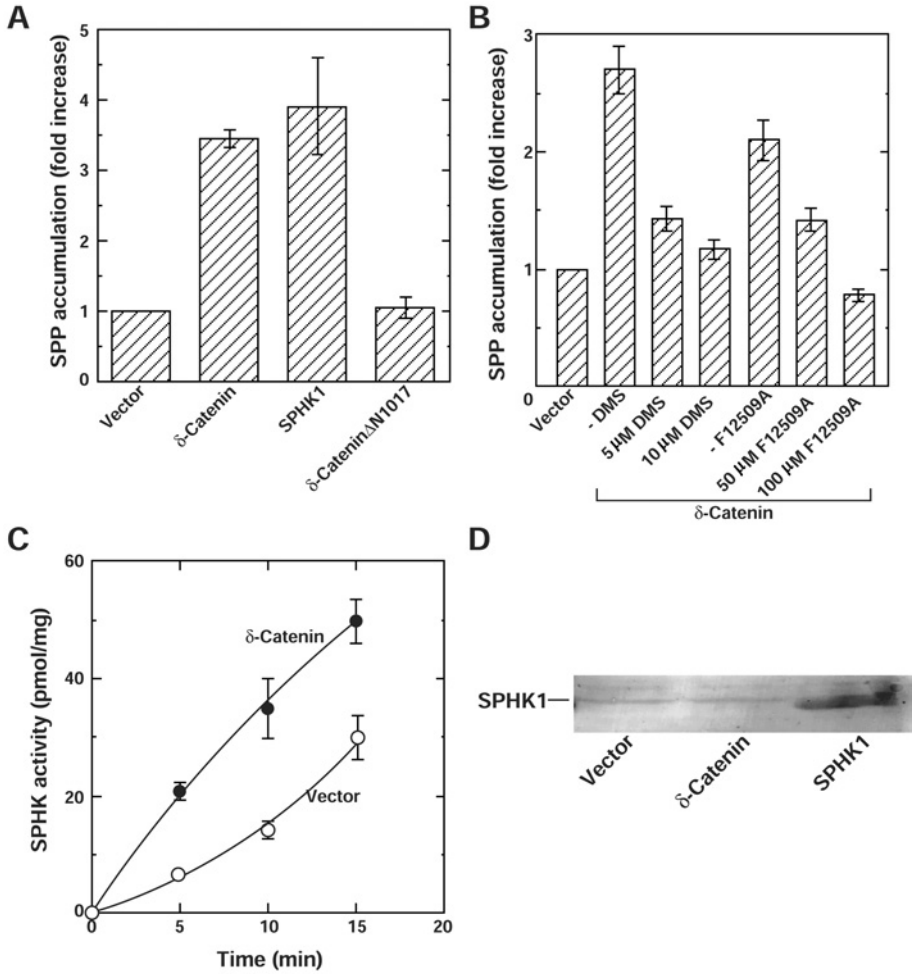

Figure 3 SPP accumulation in δ-catenin/NPRAP-expressing MDCK cells

(**A**) MDCK cells stably transfected with δ-catenin/NPRAP, δ-catenin(ΔN1017), SPHK1 or empty vector were metabolically labelled with [^{32}P]orthophosphate and intracellular [^{32}P]SPP was quantified. (**B**) Various concentrations of DMS, F12509A or vehicle (0.01 % ethanol for DMS and 0.1 % DMSO for F12509A) were added to the culture medium and cellular [^{32}P]SPP content was quantified. The results are expressed as a fold increase over control (SPP level in the cells with empty vector) and are the means ± S.E.M. from three independent experiments done in triplicate. (**C**) Post-nuclear supernatants (50 μg each) prepared from MDCK cells stably transfected with either δ-catenin/NPRAP (●) or empty vector (○) were assayed for SPHK activity for the indicated times. (**D**) Cell lysates from MDCK cells stably expressing δ-catenin/NPRAP, SPHK1 or empty vector (50 μg of protein each) were subjected to SDS/PAGE followed by immunoblotting with anti-SPHK1 antibody. δ-Catenin, δ-catenin/NPRAP.

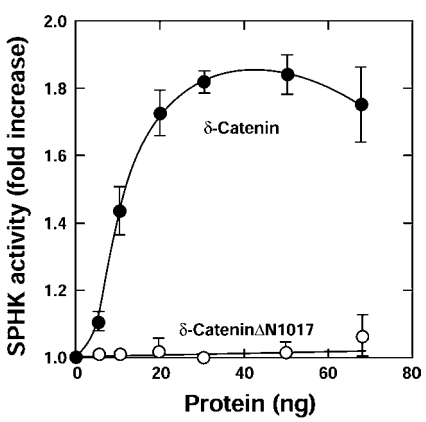

Figure 4 δ-Catenin/NPRAP stimulates SPHK1 activity *in vitro*

SPHK1 activity was measured as a function of δ-catenin/NPRAP or δ-catenin(ΔN1017) concentrations as indicated. The results are expressed as a fold increase and are the means ± S.E.M. from three independent experiments carried out in triplicate.

So far, tumour necrosis factor α receptor-associated factor 2 [35], protein kinase A anchoring protein-related protein [36], and RPK118 [26] have been identified as SPHK1-interacting molecules. In the present study we have shown that δ-catenin/NPRAP specifically interacts with SPHK1 from the results of (a) yeast two-hybrid screening (Figure 1), (b) co-immunoprecipitation assay (Figure 1) and (c) immunofluorescence studies (Figure 2). δ-Catenin/NPRAP has no obvious structural similarity with these SPHK1-interacting molecules. Diverse biological effects of intracellular SPP may be explained by the variety of interacting partners for SPHK1, an enzyme with the highest efficiency for the production of SPP [16].

δ-Catenin/NPRAP is known to possess pleiotropic functions, including enhancement of cell scattering upon stimulation with HGF and organization of cell–cell junctions by interacting with E-cadherin and β-catenin [20]. In the present studies we have demonstrated that δ-catenin/NPRAP-induced enhancement of cell migration in MDCK cells is mediated by the SPHK1–SPP signalling pathway from the observations that (a) δ-catenin/NPRAP specifically interacts with SPHK1 (Figure 1), (b) δ-catenin/NPRAP

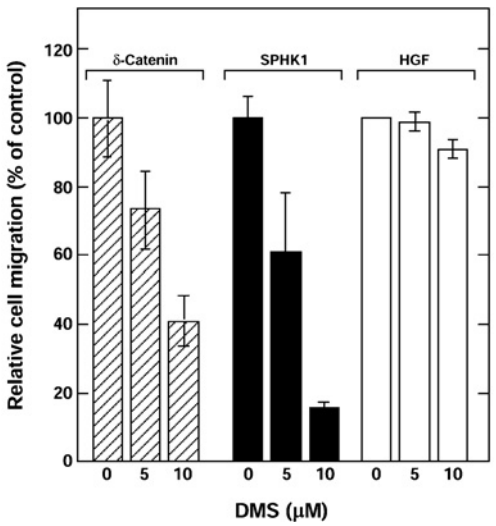

Figure 5 Inhibition of δ-catenin/NPRAP-induced cell migration by DMS

Effect of DMS on cell migration in MDCK cells stably expressing δ-catenin/NPRAP or SPHK1 was measured using a two-chamber 96-well system. Various concentrations of DMS as indicated were included in the lower chamber during cell migration analyses. Effect of DMS was also measured in HGF-stimulated mock-treated MDCK cells. The results are expressed as a percentage of control (cell numbers that migrated from the upper to the lower chambers in the absence of DMS) and are the means ± S.E.M. from three independent experiments carried out in triplicate. δ-Catenin, δ-catenin/NPRAP.

activates SPHK1 in a purified cell-free system (Figure 4), (c) cellular SPP accumulates in MDCK cells stably expressing δ-catenin/NPRAP (Figure 3), and (d) SPHK inhibitor DMS selectively inhibits δ-catenin/NPRAP-induced cell migration (Figure 5).

In contrast to the expression of δ-catenin/NPRAP, which is restricted to the neuronal tissues, SPHK1 is known to be ubiquitously distributed among all tissues [15]. It is, therefore, important to address whether the specific interaction of δ-catenin/NPRAP with SPHK1 is valid only for cells expressing δ-catenin/NPRAP (brain) or if other widely distributed protein(s), which are structurally related to δ-catenin/NPRAP behave similarly. In this context we have recently observed that $p120^{ctn}$, a ubiquitously expressed catenin subfamily with 10 armadillo repeats, also interacts specifically with SPHK1 (results not shown). In order to generalize the mechanism of action of $p120^{ctn}$ subfamily proteins via the SPHK–SPP signalling pathway, further studies, including interaction of SPHK1 with other $p120^{ctn}$ subfamily members, will be necessary.

We observed good co-localization of endogenous δ-catenin/NPRAP with SPHK1 in dissociated rat hippocampal primary neurons (Figure 2). Recently, a potential functional connection between δ-catenin/NPRAP and the synaptic signalling pathway during neuronal development has been suggested. For example, it has been reported that the expression of δ-catenin/NPRAP induces the branching of dendrite-like processes in NIH 3T3 cells and enhances dendritic morphogenesis in primary hippocampal neurons [37]. It is also interesting to note that the hemizygous loss of δ-catenin/NPRAP is associated with severe mental retardation in Cri-du-Chat syndrome [38]. Intriguingly, the C-terminal deletion mutant of δ-catenin/NPRAP observed in human Cri-du-Chat patients is shown to cluster and redistribute the full-length protein and dominantly inhibit its branch-inducing effect [37,38]. The δ-catenin/NPRAP(ΔC209) mutant used in the present study, which resembles the mutant in human Cri-du-Chat patients [38], possesses the capacity to bind to SPHK1 (Figure 1B). Studying the involvement of SPHK1 in the structural remodelling of synapses during neuronal development will be an interesting research enterprise.

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