Stimulation of nuclear sphingosine kinase activity by platelet-derived growth factor

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Abstract Subcellular fractionation revealed that a significant fraction of total sphingosine kinase, the enzyme that phosphorylates sphingosine to form the bioactive lipid metabolite sphingosine-1-phosphate, resides in the nuclei of Swiss 3T3 cells, localized to both the nuclear envelope and the nucleoplasm. Platelet-derived growth factor, in addition to rapidly stimulating cytosolic sphingosine kinase, also induced a large increase in nucleoplasm-associated activity after 12–24 h that correlated with progression of cells to the S-phase of the cell cycle and translocation of sphingosine kinase–green fluorescent protein fusion protein to the nuclear envelope. Our results add sphingosine kinase to the growing list of lipid-metabolizing enzymes associated with the nucleus, and suggest that sphingosine-1-phosphate may also play a role in signal transduction in the nucleus. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Nuclear sphingosine kinase; Sphingosine; Sphingosine-1-phosphate

1. Introduction

The canonical model of lipid second-messenger action involves the generation of bioactive lipid metabolites at the plasma membrane that activate signaling cascades, leading to the stimulation of activities in target organelles, including the nucleus. However, it has become apparent that the nuclear envelope is also an active participant in signaling cascades involving induction of specific types of nuclear lipid metabolism [1].

Although still somewhat controversial, a substantial body of literature has implicated ceramide and sphingosine in cell growth arrest and apoptosis induced by cytokines, ionizing radiation, anti-cancer drugs, growth factor withdrawal, and various other apoptotic agents (reviewed in [2]), while a further metabolite, sphingosine-1-phosphate (SPP), generally promotes cell growth and survival [3]. Numerous studies have demonstrated that activation of either neutral or acidic sphingomyelinase plays an important role in apoptosis [4,5]. The observation that the composition and turnover of sphingomyelin in the internal nuclear matrix changes during hepatocyte maturation [6] together with the detection of neutral sphingomyelinase activity in both the nuclear membrane and the chromatin fractions [7], led to the suggestion of a potential role for nuclear sphingomyelin metabolites [8,9]. In agreement with an apoptotic function of ceramide, activation of nuclear sphingomyelinase leading to accumulation of ceramide and sphingosine occurs after portal vein branch ligation, a procedure which induces apoptosis of hepatocytes [10]. Moreover, neutral sphingomyelinase activation, ceramide generation, and apoptotic features (PARP cleavage, nuclear fragmentation, DNA laddering) were observed only in highly purified nucleus preparations from radio-sensitive, but not in radio-resistant, myeloid leukemic cells [11]. These observations suggest that nuclear sphingomyelinase and ceramide formation may contribute to ionizing radiation-triggered apoptosis [11]. Intriguingly, sphingomyelinase activity present in the chromatin fraction increases during liver regeneration after partial hepatectomy as cells progress through the S-phase of the cell cycle [12], further supporting the notion that there is an intranuclear sphingomyelin cycle and that nuclear sphingolipid metabolites may play a regulatory role in mediating cell growth.

Of interest, sphingosine kinase (SPHK), the enzyme that catalyzes the phosphorylation of sphingosine, is activated by growth and survival factors, including platelet-derived growth factor (PDGF), serum [13], nerve growth factor [14], and vitamin D3 [15], as well as activation of protein kinase C [16], resulting in increased intracellular levels of SPP. Abundant evidence indicates that SPP promotes cell growth, proliferation, and resistance to apoptosis [17,18]. Moreover, it has been suggested that balance between the sphingolipid metabolites ceramide/sphingosine and SPP may influence cell death and survival [16,19].

Recently, two isoforms of mammalian sphingosine kinase (SPHK1 and SPHK2) have been cloned and characterized [20,21]. Overexpression of SPHK1 induces cell proliferation by promoting the G1 to S-phase transition of the cell cycle, as well as inhibiting the apoptotic response to serum deprivation of ceramide [22]. Moreover, cells overexpressing SPHK1 acquire a transformed phenotype, as determined by focus formation, colony growth in soft agar and the ability to form tumors in NOD/SCID mice [23]. It is of interest that another

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Abbreviations: BSA, bovine serum albumin; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; SPHK, sphingosine kinase; SPP, sphingosine-1-phosphate

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lipid kinase, diacylglycerol kinase ξ, localizes to the nucleus and plays a role in signal transduction [24,25]. Thus, we examined the possibility that SPHK, acting similarly to diacylglycerol kinase, may be part of a lipid-signalling system in the nucleus. Here we report that a significant fraction of total cellular SPHK activity resides in the nuclei of Swiss 3T3 cells. Moreover, the rapid increase in SPHK activity following stimulation of PDGF that occurs after 5–10 min is followed by a large increase in nuclear SPHK activity at much later times, which correlates with progression of cells to the S-phase of the cell cycle.

2. Materials and methods

2.1. Materials

Human PDGF-BB was purchased from Calbiochem (San Diego, CA, USA), SPP and sphingosine were from Biomol Research Laboratory Inc. (Plymouth Meeting, PA, USA). [γ-^{32}P]ATP (3000 Ci/mmol) was from Amersham (Arlington Heights, IL, USA). Serum and medium were obtained from Biofluids, Inc. (Rockville, MD, USA). Insulin and transferrin were from Collaborative Research (Lexington, MA, USA) and silica gel 60 G plates from EM Sciences (Cherry Hill, NJ, USA). Bovine serum albumin (BSA) and reagents for lactate dehydrogenase and α-glucosidase II assays were from Sigma (St. Louis, MO, USA).

2.2. Cell culture

Swiss 3T3 and NIH 3T3 fibroblasts were obtained from American Type Culture Collection (Rockville, MD, USA) and cultured as previously described [26]. Swiss 3T3 fibroblasts were seeded at a density of 1.5×10^4 cells/cm^2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% calf serum, refed after 2 days, and were used 5 days later when confluent and quiescent. For stimulation with PDGF, cells were washed twice with phosphate-buffered saline (PBS) and cultured in serum-free medium containing insulin (2 μg/ml), transferrin (4 μg/ml), and BSA (20 μg/ml). Cells were then stimulated with PDGF (10 ng/ml) or vehicle for the indicated times.

2.3. Isolation of nuclei

Two methods were employed to purify intact nuclei from cells. In each method, light microscopy was used to confirm that efficient cell lysis had occurred (greater than 90% in all cases).

2.3.1. Method I. This method for separating nuclei from cells was carried out essentially as described [27], with the exception that Tween-40 was omitted from the extraction buffer. Briefly, cells were washed with PBS, scraped in PBS, and then pelleted at $5000\times g$ for 5 min. Cells were resuspended in 400 μl buffer A (10 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 15 mM KCl, 1 mM phenylmethylsulfonyl fluoride (PMSF)) at 5×10^6 cells/ml, frozen in liquid nitrogen, thawed rapidly at 37°C, and then passed through a 25-gauge needle 15 times. The resulting homogenate was layered on top of 200 μl of a sucrose cushion (50% sucrose in buffer A) and centrifuged at $15\,000\times g$ for 3 min. Nuclei-free cytoplasm was removed from above the sucrose layer. Only intact nuclei pelleted through the cushion, as confirmed by light microscopy.

2.3.2. Method II. Nuclei were also isolated by minor modifications of a method described by Dignam et al. [28]. Cells were suspended in 5 volumes buffer B (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF, 0.5 mM dithiothreitol). After 15 min on ice, cells were lysed by passing five times through a 25-gauge needle. Nuclei were collected by centrifugation at $800\times g$ for 5 min. The supernatant was then centrifuged at $16\,000\times g$ for 10 min, and the resulting supernatant designated as the nuclei-free cytoplasm.

2.4. Fractionation of nuclei

The nuclei obtained from methods I and II were further fractionated into membrane and soluble components. Intact nuclei were resuspended in 200 μl buffer A and sonicated three times at 200 W for 20 s, and then centrifuged at $16\,000\times g$ for 10 min to separate the nucleoplasm (supernatant) from the nuclear envelope and matrix (pellet). The nucleoplasm was subjected to an additional centrifugation at $100\,000\times g$ for 30 min, and the resulting supernatant was designated as the S-100 fraction.

To determine the purity of subcellular fractions, the activities of specific marker enzymes for cytosol and endoplasmic reticulum (ER), lactate dehydrogenase and α-glucosidase II, respectively, were measured. Lactate dehydrogenase activity was measured according to the supplier's protocol (Sigma). α-Glucosidase II activity was determined as described [29], with minor modifications. Briefly, 4-methylumbelliferyl-α-D-glucoside was used as substrate in 100 mM phosphate buffer, pH 7.0, containing 0.1% Triton X-100, and assays were carried out for 15 min at 37°C. Reactions were terminated by addition of ice-cold 250 mM potassium glycine, pH 10.3, and the release of 4-methylumbelliferone was measured with a spectrofluorimeter (365 nm excitation, 450 nm emission).

2.5. Measurement of SPHK activity

SPHK activity in cytosolic or nuclear fractions was determined with 50 μM sphingosine, added as a complex with BSA (4 mg/ml), [γ-^{32}P]ATP (10 μCi, 1 mM) containing $MgCl_2$ (10 mM), as previously

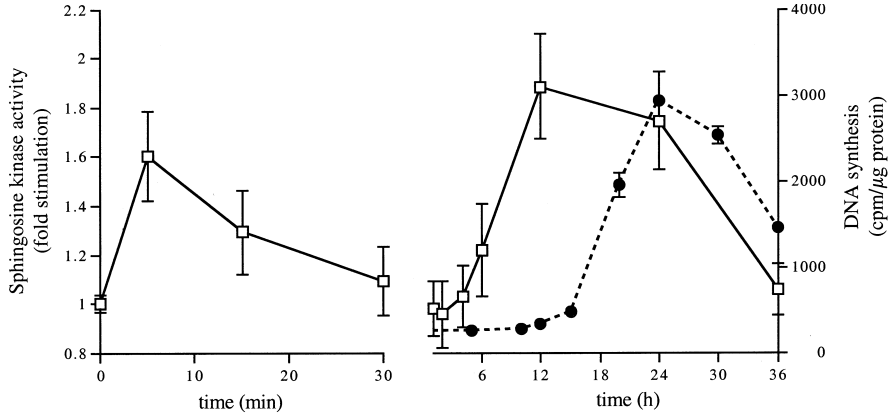

Fig. 1. Effect of PDGF on SPHK and DNA synthesis. Confluent and quiescent cultures of Swiss 3T3 cells were washed in DMEM and incubated in DMEM supplemented with BSA (20 μg/ml), transferrin (5 μg/ml), and insulin (4 μg/ml) in the absence or the presence 10 ng/ml PDGF. SPHK activity in cell lysates was measured after short- and long-term treatments with PDGF (open squares). Data are the mean ± S.D. of triplicate determinations and are expressed as fold stimulation relative to untreated controls. In duplicate cultures, PDGF-treated cultures were pulsed with [^{3}H]thymidine 2 h prior to the indicated times, and incorporation into DNA was measured (filled circles). Data are expressed as cpm incorporated per μg protein. It should be noted that treatment of cells with insulin and transferrin had no effect on SPHK activity or on DNA synthesis.

described [20]. Specific activity is expressed as pmol SPP formed per min per mg protein.

2.6. Measurement of DNA synthesis

Cells were pulsed for 2 h with 1 μCi of [^{3}H]thymidine and incorporation of radioactivity into trichloroacetic acid-insoluble material was measured as described [30]. Standard deviations were routinely less then 10% of the mean.

2.7. Visualization of SPHK–green fluorescent protein (GFP)

NIH 3T3 fibroblasts were plated on glass coverslips that had been coated with 50 μg/ml collagen and then transfected with 1 μg of GFP–SPHK1 fusion plasmid as described [22]. Cells were washed twice with serum-free medium and starved for 3 h in serum-free medium containing insulin (2 μg/ml), transferrin (4 μg/ml), and BSA (20 μg/ml). Cells were then treated with vehicle or 10 ng/ml PDGF at 37°C for 24 h and fixed with 3.7% formaldehyde in PBS for 20 min. Confocal fluorescent and Nomarski images were collected using an Olympus microscope.

3. Results and discussion

3.1. Biphasic effects of PDGF on SPHK activity

Previously, we found that PDGF rapidly stimulates SPHK activity in Swiss 3T3 cells with subsequent increases in cellular SPP levels [31]. Moreover, inhibition of SPHK by a competitive inhibitor blocked PDGF-induced DNA synthesis, suggesting that SPHK activation is important for the mitogenic

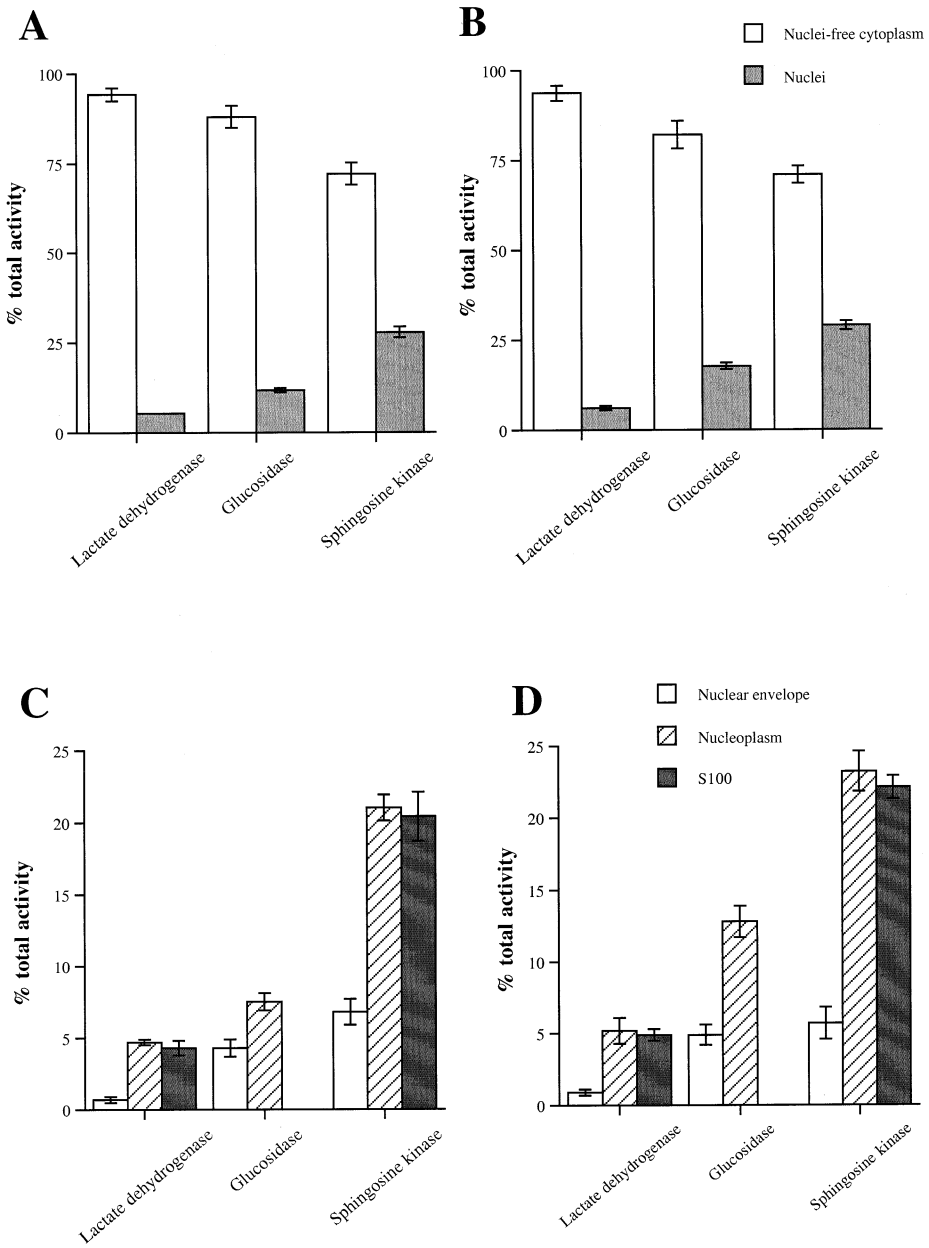

Fig. 2. Nuclear SPHK. Nuclei were purified from confluent and quiescent 3T3 fibroblasts by methods I (A) or II (B) as described in Section 2, and SPHK, lactate dehydrogenase and α-glucosidase II activities were measured in the nuclei-free cytoplasm (open bars) and purified nuclei (filled bars). Nuclei obtained from method I (C) or II (D) were further fractionated into nuclear envelope (open bars) and nucleoplasm (hatched bars) and S-100 (filled bars) fractions, and SPHK, lactate dehydrogenase and α-glucosidase II activities measured. Data are expressed as percent of total activity and are the mean ± S.D. of triplicate determinations. α-Glucosidase II activity was below detection limits in the S-100 fraction. Similar results were obtained in three independent experiments.

activity of PDGF [31]. The increase in SPHK activity observed was transient, with activity returning to basal levels within 1 h. We have now examined the effects of PDGF on SPHK activity over a much longer period. Interestingly, PDGF induced a biphasic activation of SPHK. In agreement with earlier studies [31], we observed a rapid and transient 1.7-fold increase in SPHK activity, peaking at 5–10 min and declining to control levels within 30 min (Fig. 1A). PDGF also induced a second, two-fold increase of SPHK activity, peaking at about 12–24 h after stimulation, which was more sustained and only returned to basal levels after 36 h (Fig. 1B). We also examined the time course of the increase in DNA synthesis. PDGF significantly increased DNA synthesis in quiescent cultures of Swiss 3T3 cells after 18 h of exposure, reached a maximum after 24 h, and declined thereafter (Fig. 1B). This is consistent with numerous studies demonstrating that there is a lag period of around 15 h preceding the entry into the S-phase of the cell cycle in response to growth factors (reviewed in [32]).

3.2. Nuclear SPHK activity

Recently, activation of nuclear sphingomyelinase, which hydrolyzes sphingomyelin to ceramide, has been implicated in ionizing radiation-induced cell death in certain myelocytic cell lines [11]. Because ceramide can be further metabolized to sphingosine, the substrate for SPHK, it was important to determine whether there might also be nuclear-associated SPHK activity. To examine this possibility, intact nuclei were isolated from cells by two different, non-detergent-based methods. To rigorously confirm the purity of the nuclei, assays of marker enzymes specifically localized in the cytosol (lactate dehydrogenase) or in the ER (α-glucosidase II) were performed. Both nuclear preparation methods effectively removed cytosolic contamination, with less then 6% of lactate dehydrogenase activity found in the purified nuclei. However, method I was more effective at removing endoplasmic reticulum contamination, as measured by α-glucosidase II activity, with 9% contamination by this method versus 18% contamination for method II (Fig. 2B). Therefore, method I was used in subsequent experiments, though method II gave substantially the same results for SPHK activity in nuclear fractions. Interestingly, when SPHK activity in the subcellular fractions was measured, approximately 30% of the total cellular SPHK activity was found to be associated with the nucleus. This is unlikely to be due to cytosolic contamination as shown by the lactate dehydrogenase assays (Fig. 2A,B). Although it is difficult to absolutely exclude the presence of contaminating membranes in nuclei preparations, especially considering the continuous nature of the nuclear envelope with the ER, the absence of ER-specific α-glucosidase II activity revealed that the presence of SPHK in the nucleus cannot be explained by contamination with endoplasmic membrane.

As this is the first demonstration of the occurrence of SPHK activity in the nucleus, it was important to determine whether the SPHK activity was associated with the nuclear envelope or in the nucleoplasm. To this end, purified nuclei were further fractionated. Most of the nuclear SPHK activity ($>75\%$) was found in the nucleoplasm. One-quarter of the SPHK activity was found in the insoluble nuclear fraction, suggesting that some SPHK is either tightly bound to membranes or the nuclear matrix (Fig. 2C,D). To rule out the possibility that the soluble nuclear fraction was contaminated with a specialized subcompartment of the ER, the soluble nuclear fraction was further centrifuged at $100\,000 \times g$ to pellet all membranes. All of the SPHK activity remained soluble, while all of the ER marker, α-glucosidase II, was pelleted (Fig. 2C,D). These results suggest that SPHK is not associated with ER membranes, but rather is localized to the nucleoplasm.

3.3. Differential effects of PDGF on nuclear SPHK activity

The finding that nuclei contain a relatively high basal

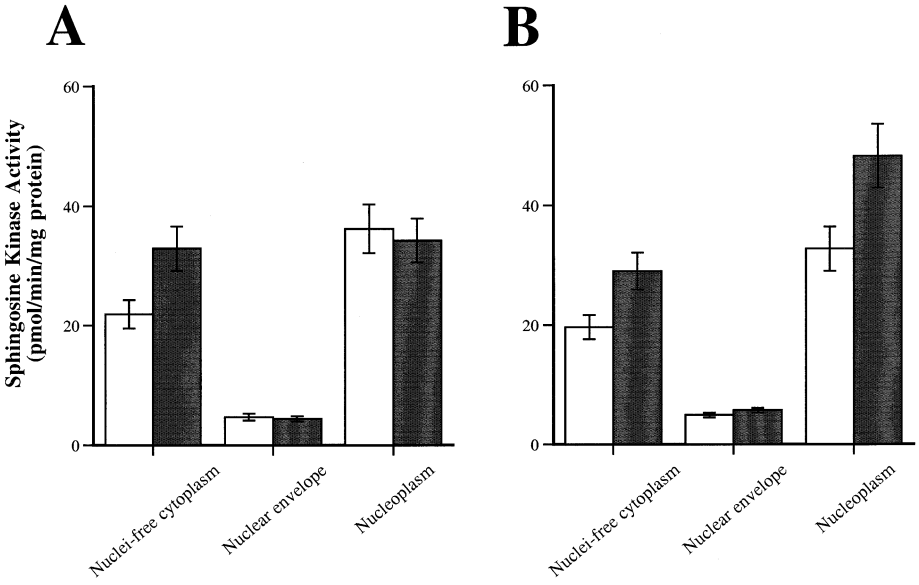

Fig. 3. Effects of PDGF on nuclear SPHK activity. Confluent and quiescent 3T3 fibroblasts were treated without (open bars) or with 10 ng/ml PDGF (filled bars) for either 10 min (A) or 24 h (B). The cells were then harvested and nuclei prepared by method I. SPHK activity was measured in nuclei-free cytosol, nuclear envelope, and nucleoplasmic fractions. Data are the mean ± S.D. of triplicate assays and are expressed as percent of total activity. Similar results were obtained in three independent experiments.

SPHK activity, raised the possibility that nuclear SPHK activity might also be regulated by PDGF. Thus, cells were stimulated with PDGF for 10 min or 24 h and SPHK activity determined in purified nuclei. As expected, treatment with PDGF for 10 min stimulated SPHK activity in the nuclei-free cytoplasm by 1.6-fold (Fig. 3A). However, no detectable changes in either nuclear envelope or nucleoplasmic SPHK activity were detected at this short time period. In contrast, after 24 h of treatment, at a time during the second peak of SPHK activity, there was a significant increase in SPHK activity in both nuclei-free cytoplasm and nucleoplasm. These data suggest that during progression of cells through the S-phase of the cell cycle, stimulation of SPHK or translocation to the nucleus might play an important role.

3.4. Translocation of SPHK1 to the nucleus

We previously found that when cells were transfected with SPKH1, the recombinant enzyme was stimulated to the same extent by brief treatment with PDGF as endogenous SPHK [22]. However, the effects of PDGF stimulation on SPHK activity at the longer time points corresponding to the second peak of activity were not previously examined. We have now used a SPHK1–GFP fusion protein to determine whether SPHK1 is translocated to the nucleus after stimulation with PDGF. In agreement with our previous study with c-myc-tagged SPHK1 [22], SPHK1–GFP was diffusely distributed in the cytosol of 3T3 cells, with somewhat denser expression in perinuclear sites. After 24 h treatment with PDGF, there was a noticeable translocation of the SPHK1–GFP fusion protein to the nuclear envelope (Fig. 4), as well as changes in the fine-granular distribution in the cytosol. SPHK1 has no signal sequence or transmembrane domains, and can only peripherally associate with the nuclear envelope. It should be noted that disruption or purified nuclei released more than 75% of the nuclear SPHK activity to the supernatant, suggesting that SPHK1 is peripherally associated with the inner nuclear membrane and that this interaction is disrupted during fractionation of the nuclei into nucleoplasm, nuclear envelope and matrix.

In conclusion, we present evidence here for the existence of a significant pool of nuclear SPHK that increases upon stimulation with the potent mitogen, PDGF. These observations suggest a potential role for SPHK-dependent SPP synthesis in nuclear-signalling pathways. This is a provocative suggestion, as it is now well accepted that the main cellular targets of SPP are receptors that reside in the plasma membrane. Abundant evidence indicates that SPP is the ligand for G-protein-coupled receptors, known as the EDG-1 family. To date, five different members have been identified, EDG-1, -3, -5, -6, and -8 [17,33,34]. These receptors are developmentally regulated, highly expressed in the cardiovascular and nervous systems, and are coupled to a variety of G-proteins, and thus

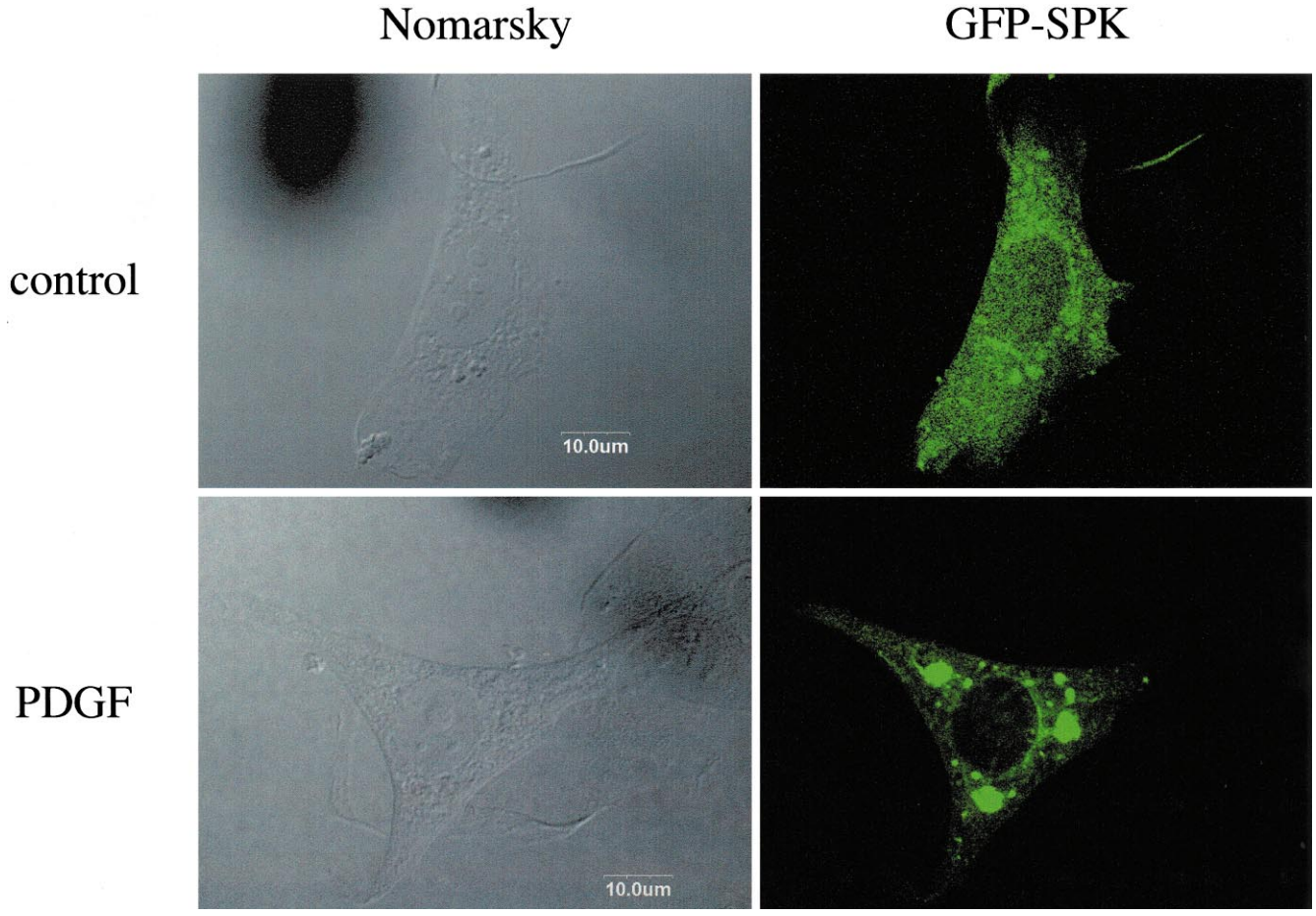

Fig. 4. Cellular localization of SPHK1–GFP fusion protein. NIH 3T3 fibroblasts were transiently transfected with SPHK1–GFP and after 24 h, stimulated without or with 10 ng/ml PDGF for 24 h. Cells were examined by Nomarski to visualize the nucleus and by fluorescence microscopy to localize the SPHK1–GFP fusion protein. GFP–SPHK1 was found to have identical enzymatic properties as the native enzyme.

regulate diverse biological processes [17,18,33,34]. The existence of sphingomyelin metabolites [7,8,11] and SPHK in the nucleus suggests independent functions of SPP in the nucleus. The physiological significance of nuclear SPHK and its product SPP, as well as the potential targets of SPP in the nucleus is an important area for future investigation.

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