An oncogenic role of sphingosine kinase

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Sphingosine kinase (SphK) is a highly conserved lipid kinase that phosphorylates sphingosine to form sphingosine-1-phosphate (S1P). S1P/SphK has been implicated as a signalling pathway to regulate diverse cellular functions [1–3], including cell growth, proliferation and survival [4–8]. We report that cells overexpressing SphK have increased enzymatic activity and acquire the transformed phenotype, as determined by focus formation, colony growth in soft agar and the ability to form tumours in NOD/SCID mice. This is the first demonstration that a wild-type lipid kinase gene acts as an oncogene. Using a chemical inhibitor of SphK, or an SphK mutant that inhibits enzyme activation, we found that SphK activity is involved in oncogenic H-Ras-mediated transformation, suggesting a novel signalling pathway for Ras activation. The findings not only point to a new signalling pathway in transformation but also to the potential of SphK inhibitors in cancer therapy.

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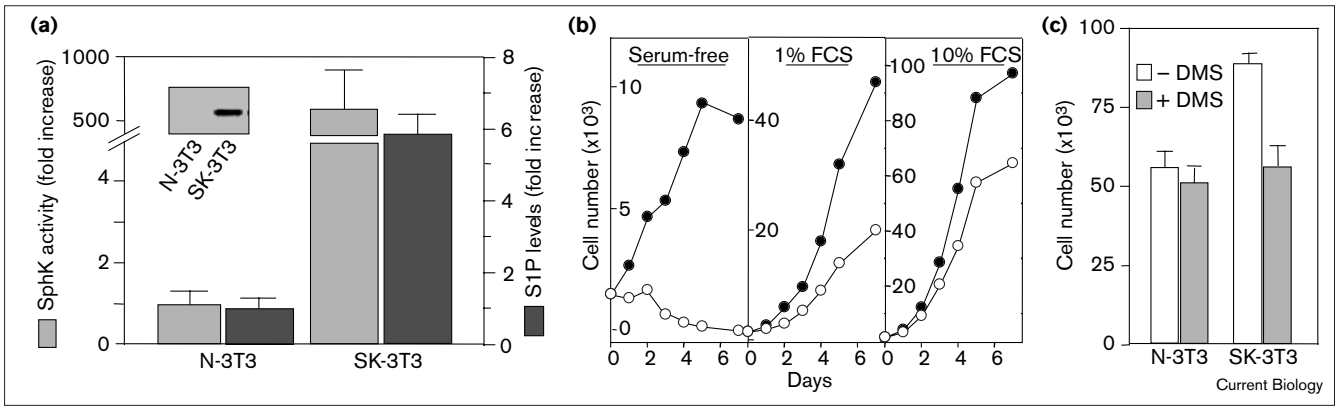
Results and discussion

To investigate the oncogenic role of SphK, non-transformed NIH3T3 fibroblasts were transfected with human SphK cDNA, which was recently cloned in our laboratory [9]. Pooled stable transfectants (referred to as SK-3T3) were used to avoid the phenotypic artefacts that may arise from the selection and propagation of individual clones from single transfected cells. In the SK-3T3 cell pools, SphK activity was increased by over 600-fold (Figure 1a) in comparison to NIH3T3 cells that were transfected with the empty vector (N-3T3). Immunoblot analysis showed a specific protein band with an apparent molecular weight consistent with the predicted size of FLAG epitope-tagged human SphK, which was detected only in the SK-3T3 pools but absent in N-3T3 cells (Figure 1a, inset). Intracellular levels of S1P, the direct product of SphK, were increased in SK-3T3 cells by 5.9 ± 0.9-fold, indicating that SphK was activated constitutively in the stable transfectants (Figure 1a). Although there was a wide range of SphK expression levels (increases of between 12-fold and ~800-fold) in different clones of transfected cells, intracellular levels of S1P were increased by only between 4-fold and ~8-fold. Thus, S1P levels did not correlate with the expression levels of SphK, assayed by *in vitro* enzyme activity. This may have been an influence of the availability and/or subcellular location of sphingosine, and rapid degradation of S1P by S1P phosphatase or S1P lyase. Despite notable increases in intracellular levels of S1P, there was no detectable S1P secretion into the extracellular media (data not shown), which is consistent with a previous report showing that overexpression of SphK in NIH3T3 or HEK293 cell lines were unable to secrete S1P [8].

The growth curves of SK-3T3 cells were significantly different from those of control cells (Figure 1b). Overexpression of SphK dramatically enhanced cell growth in media containing either 1% or 10% serum. Even in serum-free medium for up to 7 days, SK-3T3 cells survived and grew whereas the N-3T3 cells were dying. Furthermore, when the cells reached saturation density, SK-3T3 cells continued to proliferate (see Supplementary material), suggesting an escape from contact inhibition. Treatment of SK-3T3 cells with a specific inhibitor of SphK, *N*,*N*-dimethylsphingosine (DMS), significantly diminished the enhanced proliferation induced by overexpression of SphK, whereas DMS had no effect on proliferation of N-3T3 cells (Figure 1c). These results suggest that the constitutive activation of SphK in cells stably transfected with SphK reduces two key growth-limiting properties: serum dependence and contact inhibition.

The transforming activity assayed by focus formation in NIH3T3 cells showed that cells transfected with SphK but not empty vector induced numerous foci (Table 1 and Figure 2a). Both SphK and control vectors displayed similar efficiency in the generation of G418-resistant colonies (data not shown), indicating that the transforming activity was not a non-specific effect of transfection. Furthermore, SK-3T3 cells formed vigorous colonies in soft agar (Table 1 and Figure 2b), revealing the acquisition of anchorage-independent growth. Although N-3T3 cells exhibited a low background level of colony formation, which may be due to spontaneous transformation,

Figure 1



Overexpression of SphK stimulates proliferation in NIH3T3 cells. **(a)** Cytosolic SphK activity (light grey bars) and intracellular S1P levels (dark grey bars) were measured in the cells stably transfected with a plasmid expressing FLAG epitope-tagged SphK (SK-3T3) or empty vector alone (N-3T3) as described previously [9]. SphK activity was measured by incubating the cytosolic fraction with 5 μM sphingosine dissolved in 0.1% triton X-100 and [γ-^{32}P]ATP (1 mM, 0.5 mCi/ml) for 15 min at 37°C as described previously [16]. For assays of intracellular S1P levels, cells were labelled with [^{3}H]sphingosine (1 μM, 2 μCi/ml) for 30 min, and radioactivity incorporated into cellular lipids was extracted; [^{3}H]S1P was then resolved by thin-layer chromatography (TLC) with 1-butanol/methanol/acetic acid/water (8:2:1:2, volume/volume), visualised and quantified as described previously [8]. The inset shows expression of FLAG-tagged SphK in transfected cells by an immunoblot assay probed with anti-FLAG monoclonal antibodies (M2, Kodak). **(b)** Stably transfected SK-3T3 (shaded circles) or N-3T3 cells (unshaded circles) were plated in 48-well plates (1,000 cells per well) in DMEM containing 10% FCS. After 8 h, the cells were washed twice with DMEM and then grown in DMEM containing 1% or 10% FCS, or serum-free medium (DMEM containing 0.1% BSA). At the indicated times, cells were counted in a haemocytometer or by a thiazolyl blue (MTT) assay as described previously [7]. **(c)** Equal numbers of N-3T3 and SK-3T3 cells were plated. After a 5 day incubation in the presence or absence of DMS (2.5 μM), cell number was counted. Medium was replaced every day. Values are the mean ± SD from more than three experiments.

overexpression of SphK resulted in a 20–50-fold increase in the number of colonies and an obvious increase in colony size. Two randomly selected clones of SphK-transfected cell lines, KT-2 and KT-5, showed a similar transforming capacity although KT-2 had a 50-fold higher expression level than KT-5 (see Supplementary material). Importantly, DMS inhibited the transforming capacity of SphK in a dose-dependent manner, with 2.5 μM DMS resulting in total reversion to the normal phenotype (Figure 2b), suggesting that the activity of the enzyme is essential for the transforming process. Moreover, overexpression of a mutant SphK, SphK^{G82D}, which lacks enzymatic activity [10] failed to induce transformation (Figure 2a). Taken together, these results suggest that it is the activation of SphK, which results in increased S1P levels, rather than the overexpression itself that is responsible for the transforming capacity of this enzyme.

In addition to the intracellular function of S1P, the signalling role of SphK is also mediated by the binding of S1P to membrane G-protein-coupled receptors (Edg family), mainly in a pertussis-toxin-sensitive manner [1,2].

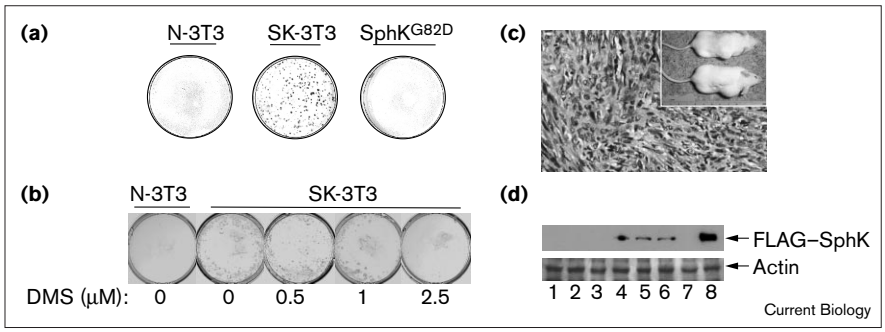
Table 1

Transforming activities and tumorigenicity in transfected NIH3T3 cells.

	Foci		Colonies in soft agar		Tumorigenicity
Cell line	– PTX	+ PTX	Number	Size (mm)	(tumours per injection)
N-3T3	1.8 ± 1.5	0.8 ± 0.6	3.3 ± 2.1	< 0.1	0/3
SK-3T3	49 ± 7.5	41.2 ± 13.2	122.3 ± 17.6	0.1–0.45	9/9

For the focus-formation assay, low-passage NIH3T3 cells were transfected with SphK (SK-3T3) or empty vector (N-3T3); 2 days later, the transfected cells were seeded to six-well plates. After reaching confluence, the cells were cultured for 3 weeks in DMEM containing 5% FCS in the presence or absence of pertussis toxin (PTX, 50 ng/ml). The foci were scored after staining with 0.5% crystal violet. For the soft-agar assay, suspensions of 1×10^4 cells from the stably transfected SK-3T3 or N-3T3 cells in a growth medium containing 0.33% agar were overlaid onto 0.6% agar gel. After 21 days incubation, colonies were stained with 0.1 mg/ml MTT and scored. Results shown are the mean ± SD from 3–5 experiments done in duplicate or triplicate. For the tumourigenesis assay, 4–6-week-old NOD/SCID mice were injected subcutaneously with 5×10^5 cells from various lines (see Supplementary material), in 200 μl sterile PBS. Animals were examined twice per week and tumours were measured after 4 weeks.

Figure 2



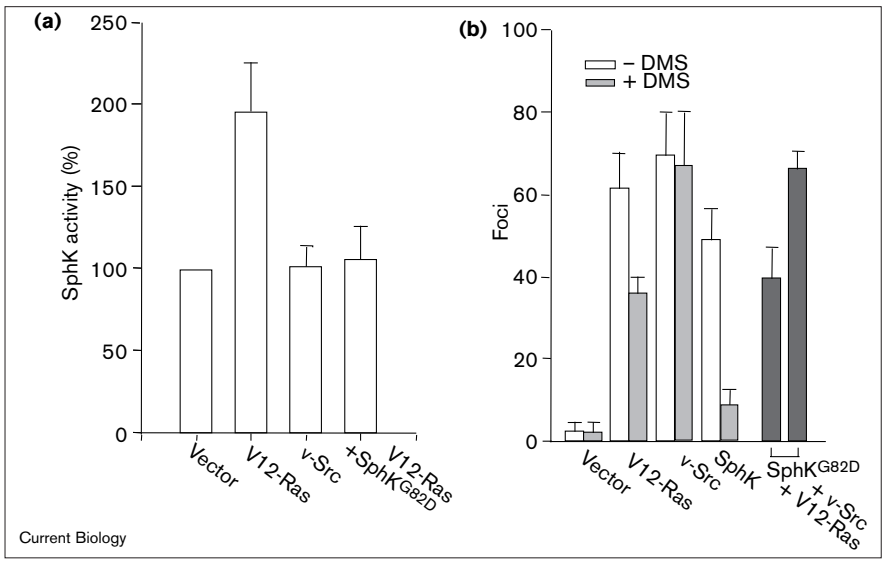
Cells overexpressing SphK are transforming and tumourigenic. **(a)** Focus-formation assay in NIH3T3 cells transfected with SphK, SphK^{G82D}, or vector alone as described in Table 1. **(b)** N-3T3 and SK-3T3 cells were cultured on soft agar and fed with medium containing various concentrations of DMS every 2 days. Colonies were stained with MTT and photographed after 3 weeks of incubation. **(c)** Morphology of a paraffin-fixed tumour section stained with hematoxylin and eosin (100 × magnification) and a photograph of tumours (inset) in NOD/SCID mice injected with SK-3T3 cells. **(d)** Whole cell extracts from three individual tumours (lanes 4–6) and respective peripheral tissues (lanes 1–3) and N-3T3 (lane 7) or SK-3T3 cells (lane 8) were analysed by western blotting. The upper blot was probed with anti-FLAG and the bottom with anti-actin antibodies.

Indeed, some of the biological responses to S1P have been reported to be inhibited by pertussis toxin [5,11,12]. However, the presence of pertussis toxin (50 ng/ml) had no effect on the transforming activity of SphK (Table 1), which is consistent with the previous report that pertussis toxin does not suppress cell growth in SphK-transfected fibroblasts [8]. Furthermore, the inhibitory effect of DMS on the transformation, along with the observation that SK-3T3 cells were unable to secrete S1P into media, do not support an extracellular role for S1P in mediating cell transformation induced by overexpression of SphK.

When SphK-transfected NIH3T3 cells from either stable transfectant pools or selected clones were injected subcutaneously into NOD/SCID mice, tumours became apparent at the site of injection within 3–4 weeks (Table 1 and Figure 2c). No mice injected with the control cells induced tumours during 10 weeks of observation. The histological appearance of tumour sections displayed the morphology of fibrosarcoma with many mitotic figures (Figure 2c). Western blot analysis of extracts derived from tumours showed high levels of FLAG-tagged protein (Figure 2d), revealing that the neoplastic cells retain and express the SphK transgenes. Thus, the tumours developed from the injected SphK-transfected cells but not from spontaneously transformed NIH3T3 cells.

Given the ability of SphK to transform cells, the potential role of SphK in the well-documented oncogene-induced transformation was explored. When NIH3T3 cells were transfected with an activated mutant H-Ras (V12-Ras), SphK activity was significantly increased to 198 ± 32% in comparison with the parental cells (Figure 3a). In contrast, cells transfected with v-Src (Figure 3a) or dominant-negative

Figure 3



SphK activation is involved in Ras transformation. **(a)** NIH3T3 cells were transfected with the indicated vectors and SphK activity was measured 48 h after transfection. **(b)** Focus-formation assays were performed in the presence or absence of DMS (2.5 μM), or in cells cotransfected with V12-Ras plus SphK^{G82D}, and v-Src plus SphK^{G82D}. Values are the mean ± SD from more than three experiments.

Ras (N17-Ras, data not shown) had no changes in SphK activity, suggesting a specific involvement of SphK in the Ras-activated pathway. Moreover, when the cells were treated with DMS, the focus formation was reduced by $41 \pm 4\%$ ($p < 0.0001$) in V12-Ras-transfected cells, but not in v-Src-transfected cells (Figure 3b), indicating that Ras transformation requires SphK activation. The inability of DMS to inhibit v-Src transformation rules out non-specific effects or a general toxicity resulting from the inhibition of SphK. To further confirm the role of SphK in Ras transformation, we used $SphK^{G82D}$, which is a dominant-negative mutant that not only lacks kinase activity but also blocks agonist-stimulated activation of endogenous SphK [10]. When V12-Ras was cotransfected with $SphK^{G82D}$, the Ras-induced SphK activation was completely inhibited (Figure 3a) and the transforming ability of Ras was also significantly reduced (Figure 3b, $p < 0.0001$). Again v-Src-induced transformation was not inhibited by $SphK^{G82D}$ (Figure 3b). Thus, SphK not only possesses transforming potential of its own right, as demonstrated by overexpression, but is also involved in Ras-mediated transformation.

The events downstream from activation of SphK that promote transformation are unclear but could include protection from apoptosis and acceleration of cell-cycle progression. Previous work has shown that increased intracellular S1P by overexpression of SphK expedites the G_1–S transition and promotes cell growth in low-serum media [8]. Agonist-stimulated endogenous SphK activation protects against cell death [5–7]. Inhibition of DEVDase activity and caspase-3 activation by SphK is likely to relate to its anti-apoptotic effect [5,6]. The effect of SphK on cell survival and growth is evolutionarily conserved [13,14], indicating the importance of SphK in regulating cell growth from yeast to mammals.

The oncogenic effect of SphK and its involvement in Ras transformation indicate a novel signalling pathway in cellular transformation and provide a potential link between sphingolipids and mammalian tumour pathogenesis. Interestingly, there is a recent report that S1P levels are significantly increased in the ascitic fluids of patients with ovarian cancer [15]. The importance of constitutive SphK activation in transformation also raises the possibility of using SphK inhibitors in cancer therapy.

Supplementary material

Supplementary material including growth of transfected cells at saturation density, transforming activity of SphK-transfected clones and a table showing tumorigenesis of NOD/SCID mice after injection of transfected cells is available at http://current-biology.com/supmat/supmatin.htm.

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