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Aminoacylase 1 is a sphingosine kinase 1-interacting protein

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Abstract Sphingosine kinase type 1 (SphK1) and its product sphingosine-1-phosphate have been shown to promote cell growth and inhibit apoptosis of tumor cells. In an effort to further understand the regulation of SphK1, we used a yeast two-hybrid screen to find SphK1-interacting proteins. One of these was identified as aminoacylase 1 (Acy1), a metalloenzyme that removes amide-linked acyl groups from amino acids and may play a role in regulating responses to oxidative stress. Both the C-terminal fragment found in the two-hybrid screen and full-length Acy1 co-immunoprecipitate with SphK1. Though both C-terminal and full-length proteins slightly reduce SphK1 activity measured in vitro, the C-terminal fragment inhibits while full-length Acy1 potentiates the effects of SphK1 on proliferation and apoptosis. Interestingly, Acy1 induces redistribution of SphK1 as observed by immunocytochemistry and subcellular fractionation. Collectively, our data suggest that Acy1 physically interacts with SphK1 and may influence its physiological functions.

Keywords: Aminoacylase 1; Sphingosine kinase; Sphingosine; Sphingosine-1-phosphate; Yeast two-hybrid

1. Introduction

Sphingolipids are ubiquitous constituents of eukaryotic membranes whose backbones consist of an acylated sphingoid base, ceramide. Ceramide and its further metabolites, sphingosine and sphingosine-1-phosphate (S1P), are now recognized as potent signaling molecules. In many cell types, increased ceramide and sphingosine levels lead to cell growth arrest and apoptosis [1,2]. Conversely, S1P promotes cell growth and inhibits apoptosis [3–5]. Cells contain enzymes that can rapidly interconvert ceramide, sphingosine, and S1P. Thus, conversion of ceramide and sphingosine to S1P simultaneously removes pro-apoptotic signals and creates a survival signal, and vice versa [6–9]. While many early studies suggested a role for S1P as an intracellular second messenger, it was later convincingly demonstrated that S1P is also a ligand for a family of G protein-coupled receptors [5,10]. Complicating matters, there is growing evidence that agonist-induced sphingosine kinase (SphK) activation leads to S1P secretion [11,12] and autocrine and/or paracrine signaling through cell surface S1P receptors [13–15].

Recently, progress has been made in elucidating the molecular mechanisms of activation of SphK type 1 (SphK1). It has been shown that PKC can phosphorylate SphK1, both activating SphK1 and inducing its translocation to the plasma membrane [12]. More recently, it has been demonstrated that activation and translocation of SphK1 from the cytosol to the plasma membrane results directly from phosphorylation at Ser225 by ERK1/2 [16]. SphK1 interacts with TRAF2, an interaction that is required for suppression of apoptosis by TNF-α [17]. Several other SphK1-interacting proteins have also recently been identified, including PECAM-1 [18], RPK118 [19], and AKAP-related protein SKIP1 [20], which are involved in the translocation of SphK1 to the plasma membrane, endosomes, and signaling complexes, respectively.

In a yeast two-hybrid search for additional SphK1-interacting proteins, we cloned aminoacylase 1 (Acy1) and showed that it interacted with SphK1 and affected its activity and biological functions.

2. Materials and methods

2.1. Cell culture and transfection

Cos7, HEK 293, and NIH 3T3 cells were obtained from ATCC. Cells were cultured in DMEM supplemented with 10% fetal bovine (Cos7, HEK) or 10% calf serum (NIH) and maintained at 37 °C in a humidified environment in 5% CO_2. All culture reagents were from BioFluids. HEK 293 cells, plated on poly-D-lysine, and NIH 3T3 cells were transfected using Lipofectamine Plus and Cos7 cells with Lipofectamine 2000 (Invitrogen).

2.2. Two-hybrid screen and cloning

The two-hybrid screen was carried out using the MatchMaker II Kit from Clontech as described [20] with mouse SphK1a as bait against a mouse kidney cDNA library (Clontech). A clone of the C-terminal portion of Acy1 (CT-Acy1) was obtained from this screen that passed all tests as a valid two-hybrid interactor. The CT-Acy1 was removed from the library vector using *Eco*RI and *Bam*H1 and cloned into pcDNA3-HA (N-terminal tag). Full-length Acy1 was cloned by PCR from a mouse kidney library using the V5-His-Topo Cloning Kit (Invitrogen).

2.3. Sphingosine kinase assay

SphK1 activity was measured essentially as described [21] with sphingosine solubilized in Triton X-100 (0.25% final concentration).

2.4. GST pulldown and immunoprecipitation

The CT-Acy1 was transcribed and translated in vitro with the TnT Kit (Promega) in the presence of [^{3}H]leucine. The translation mix was

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Abbreviations: Acy1, aminoacylase 1; S1P, sphingosine-1-phosphate; SphK1, sphingosine kinase type 1

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incubated with either GST or GST-SphK1 as described [20], then affinity-purified using glutathione–Sepharose beads (Pierce), and washed three times with SphK assay buffer containing 1% Triton X-100. The pellet was resuspended in sample buffer and proteins resolved by SDS–PAGE. Gels were dried and exposed to film. For immunoprecipitation, HEK 293 transfectants were lysed and 800 μg lysate incubated with anti-myc antibodies for 24 h at 4 °C. Anti-myc immunocomplexes were precipitated with protein A/G Sepharose (Santa Cruz) and washed three times with SphK assay buffer containing 1% Triton X-100. The pellets were resuspended in sample buffer, proteins resolved by SDS–PAGE, and immunoblotted with anti-HA (CT-Acy1) or anti-V5 (Acy1).

2.5. Apoptosis and MTT assays

48 h after transfection, NIH 3T3 cells were serum-starved for 24 h to induce apoptosis. Cells were fixed with 4% paraformaldehyde in 4% sucrose–PBS and stained with 8 μg/ml Hoechst. Apoptotic nuclei were scored essentially as described [20]. Cell viability was assessed by the MTT dye reduction assay (Roche).

2.6. Fractionation and immunofluorescence

Cells were plated on 10-cm dishes. 48 h after transfection, cells were washed and harvested in SphK buffer. Cells were lysed by freeze–thaw and then centrifuged at $100\,000 \times g$. Supernatants were removed (cytosol) and pellets washed with SphK buffer. Pellets were then resuspended in SphK buffer containing 1% Triton X-100 and solubilized on ice for 1 h. Solubilized pellets were centrifuged at $100\,000 \times g$ for 30 min and supernatants (Triton soluble, TS) and pellets (Triton insoluble, TI) were then separated. TI pellets were resuspended in SphK buffer plus 1% Triton X-100. Western blotting was used to determine protein expression with either anti-myc (9E10; Santa Cruz), anti-HA (3F10; Roche), or anti-V5 (monoclonal from Invitrogen or rabbit polyclonal from Sigma–Aldrich) as primary antibodies followed by HRP-conjugated secondary antibodies (1:10 000, Jackson ImmunoResearch Laboratories). Immunocomplexes were visualized by enhanced chemiluminescence (Pierce) as described previously [22].

For immunofluorescence, cells were plated on #1 coverslips, transfected, and after 48 h, fixed in 3.7% formalin and stained essentially as described [20]. Briefly, after washing with PBS containing 10 mM glycine, cells were permeabilized for 3 min with 0.5% Triton X-100 in PBS–glycine, washed again, and incubated for 20 min at room temperature with mouse monoclonal anti-myc (2 μg/ml) for detection of SphK1 and rabbit anti-V5 (4 μg/ml) for Acy1. After washing, cells were incubated for 20 min with Texas Red-conjugated anti-mouse and FITC-conjugated anti-rabbit secondary antibodies (1 μg/ml each; Jackson ImmunoResearch). Coverslips were then mounted with glycerol containing 10 mM *n*-propyl gallate and images collected with a Nikon TE-200 fluorescence microscope.

3. Results and discussion

3.1. Acy1 is a SphK1-interacting protein

To search for proteins that interact with SphK1 and regulate its activity or translocation to the plasma membrane, a two-hybrid screen was carried out using mouse SphK1 fused to the DNA binding domain of GAL4 as bait. The prey consisted of a mouse kidney cDNA library (Clontech) fused to the transcriptional activation domain of GAL4. Interaction between SphK1 and a library protein brings together the two domains necessary for transcription of reporter genes. The MatchMaker II system mitigates against false positives by having three different promoter–reporter gene constructs, with differing affinities for the GAL4 DNA-binding domain. This reduces the chances that the prey construct activates on its own by binding regions around the GAL4 DNA binding site or to specific TATA boxes and allows for control of stringency. Using the most stringent interaction test, a clone of the CT-Acy1, starting at amino acid 232 of the full-length protein (Fig. 1A), was obtained. Acy1 has been characterized as a

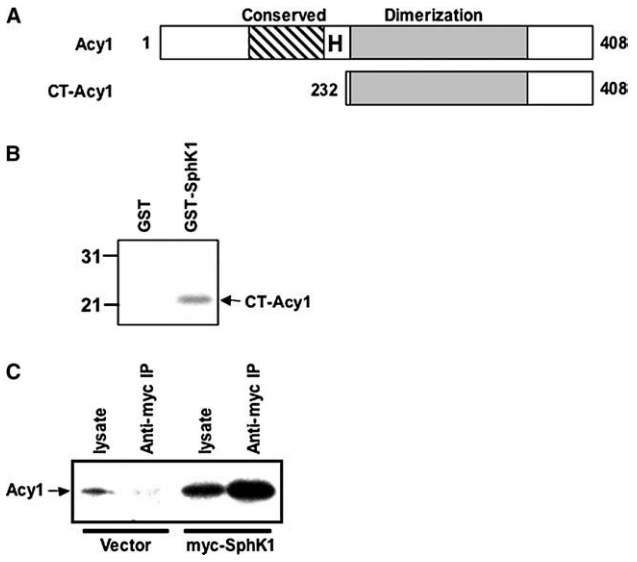

Fig. 1. SphK1 physically interacts with Acy1. (A) Schematic representation of full-length Acy1 (top) and CT-Acy1, the C-terminal fragment pulled out of the two-hybrid screen. Hatched box indicates conserved regions (aa 78–148) amongst Acy1 family members across kingdoms, H indicates the conserved catalytic histidine, and shaded boxes indicate putative dimerization domains. (B) [^{3}H]-labeled CT-Acy1 prepared by in vitro transcription–translation was incubated with either GST or GST-SphK1. Glutathione–Sepharose beads were then added. After overnight incubation at 4 °C, beads were washed and bound proteins resolved by SDS–PAGE and autoradiographed. GST-SphK1 precipitated 22 kDa radiolabeled CT-Acy1. The data are representatives of two independent experiments. (C) HEK 293 cells were co-transfected with V5-Acy1 and either vector or myc-SphK1. Cells were then lysed and immunoprecipitated with anti-myc antibodies followed by protein A/G–Sepharose. The pellets were resolved by SDS–PAGE and immunoblotted with anti-V5. Lysate indicates 1/100 of the total protein immunoprecipitated. Similar results were obtained in two additional experiments.

cytosolic homodimeric metalloenzyme of amino acid salvage [23], catalyzing the hydrolysis of amide-linked acyl chains of amino acids. It is the major acylase that degrades *N*-acetylcysteine [24], and thus may play a role in the regulation of cellular redox status. Acy1 is abundant in the kidney and brain [24], two tissues with high SphK1 levels [25]. CT-Acy1 is not expected to be active because it lacks conserved residues necessary for binding essential Zn ions and it has a truncated catalytic domain [26] (Fig. 1A).

To examine whether CT-Acy1 interacts physically with SphK1, [^{3}H]-labeled CT-Acy1 was synthesized by in vitro transcription–translation, incubated with either GST or GST-SphK1 [20] and binding was determined using glutathione–Sepharose beads. Sepharose-bound proteins were then resolved by SDS–PAGE and ^{3}H-labeled proteins visualized by autoradiography. CT-Acy1 specifically interacted with GST-SphK1, but not with GST alone (Fig. 1B).

3.2. Acy1 interacts with SphK1 in vivo

To determine if Acy1 interacts with SphK1 when expressed in mammalian cells, HEK 293 cells were co-transfected with Acy1 and SphK1. Lysates were immunoprecipitated with antibodies to SphK1 and the blots probed with antibodies to either CT-Acy1 or Acy1. Both CT-Acy1 (data not shown) and full-length Acy1 co-immunoprecipitated with SphK1

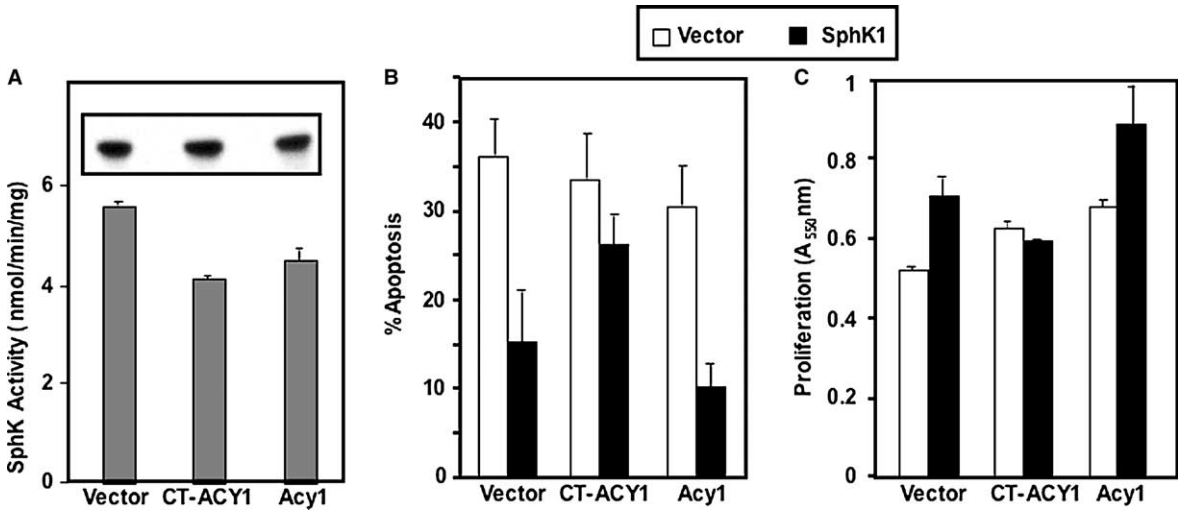

Fig. 2. Effect of Acy1 on activity and function of SphK1. (A) SphK1 activity. HEK 293 cells were co-transfected with myc-SphK1 and vector, CT-Acy1, or Acy1. After 48 h, cells were lysed and SphK1 activity measured. Inset shows equal expression of SphK1 as determined by Western blotting with anti-myc. The data are representatives of three independent experiments. (B) Cytoprotective effects of SphK1. NIH 3T3 cells stably transfected with vector (open bars) or SphK1 (filled bars) were transiently transfected with either Acy1, CT-Acy1, or empty vector, together with GFP at a 5:1 ratio, and then serum-starved. After 24 h, cells were fixed and stained with Hoechst. Total GFP-expressing cells and GFP-expressing cells displaying fragmented nuclei indicative of apoptosis were enumerated. Data are means ± S.D. Three independent wells were counted for each treatment, with a minimum of 100 cells scored per well. Data are representatives of two independent experiments. (C) Proliferative effects of SphK1. Cells transfected with the indicated constructs were plated at equal density and allowed to grow for 24 h. Cell proliferation was determined by MTT dye reduction.

(Fig. 1C). This result, coupled with the GST pull-down results and the original two-hybrid data, indicates that SphK1 and Acy1 physically interact in vivo.

3.3. Effects of Acy1 on SphK1 activity and biological functions

We next examined whether the physical interaction with Acy1 affects SphK1 biological functions. Co-transfection of SphK1 with either CT-Acy1 or Acy1 slightly decreased SphK1 activity measured in vitro, without affecting its expression level (Fig. 2A). The best characterized biological responses of SphK1 are suppression of apoptosis and stimulation of cell proliferation and entry into S phase [4,21]. NIH 3T3 cells expressing either vector or SphK1 were co-transfected with CT-Acy1 or Acy1 and effects on apoptosis induced by serum-withdrawal determined by examining chromosomal condensation and fragmentation. Interestingly, in contrast to their inhibitory effects on SphK1 activity, CT-Acy1 reduced while Acy1 potentiated the anti-apoptotic effect of SphK1 (Fig. 2B).

To address the possibility that interaction of Acy1 with SphK1 regulates its mitogenic effect, we also examined the effect of CT-Acy1 or Acy1 on proliferation. In agreement with other studies [27–30], expression of SphK1 increased cell growth as determined by MTT dye reduction assay. Once again, CT-Acy1 had a different effect than full-length Acy1. Whereas CT-Acy1 reduced the growth-promoting effect of SphK1, Acy1 enhanced it (Fig. 2C).

3.4. Acy1 induces redistribution of SphK1

SphK1 is a cytosolic enzyme, while its substrate sphingosine is a lipid found in membranes. Therefore, it is likely that SphK1 activity is regulated in part by its translocation from the cytosol to membranes. Indeed, several previous studies have shown that SphK1 translocates to membranes upon activation [12,15,16,31]. It was therefore of interest to determine whether Acy1 alters the localization of SphK1. First, we examined the localization of both proteins by immunocytochemistry. In agreement with its cytoplasmic expression [32], Acy1 had a diffuse cytosolic localization when expressed in Cos7 cells (Fig. 3A and B). When SphK1 was expressed alone, it also showed a diffuse cytosolic expression pattern (Fig. 3C and D), with dispersed punctate staining as reported previously [27]. However, when Acy1 and SphK1 were co-expressed, although both were still predominantly cytosolic, there was also co-localization in tubular structures (Fig. 3E–G, arrows) and at or near the plasma membrane as indicated by the yellow color in the merged pictures.

To further substantiate that expression of Acy1 induces redistribution of SphK1 to the plasma membrane, we examined their localization by subcellular fractionation. Transfected cells were lysed by freeze-thawing and centrifuged at $100\,000 \times g$. Pellets were then extracted with 1% Triton X-100, generating a soluble fraction and a TI fraction that contains cytoskeleton proteins, focal adhesions, and lipid rafts. As expected from the immunofluorescence data, when expressed alone, both proteins were predominantly localized to the cytosolic fraction (Fig. 4). Interestingly, when co-expressed with Acy1, a portion of SphK1 shifted from the cytosol to the TS fraction (Fig. 4).

4. Conclusions

Our results suggest that Acy1 is a bona fide SphK1-interacting protein that can influence not only its activity but also its cellular localization. Acy1 also potentiated the mitogenic and cytoprotective effects of SphK1. Surprisingly, the CT-Acy1, which also binds SphK1, reduced these effects. Although the physiological significance of these observations is not yet clear, our data suggest that CT-Acy1 may act as a dominant-

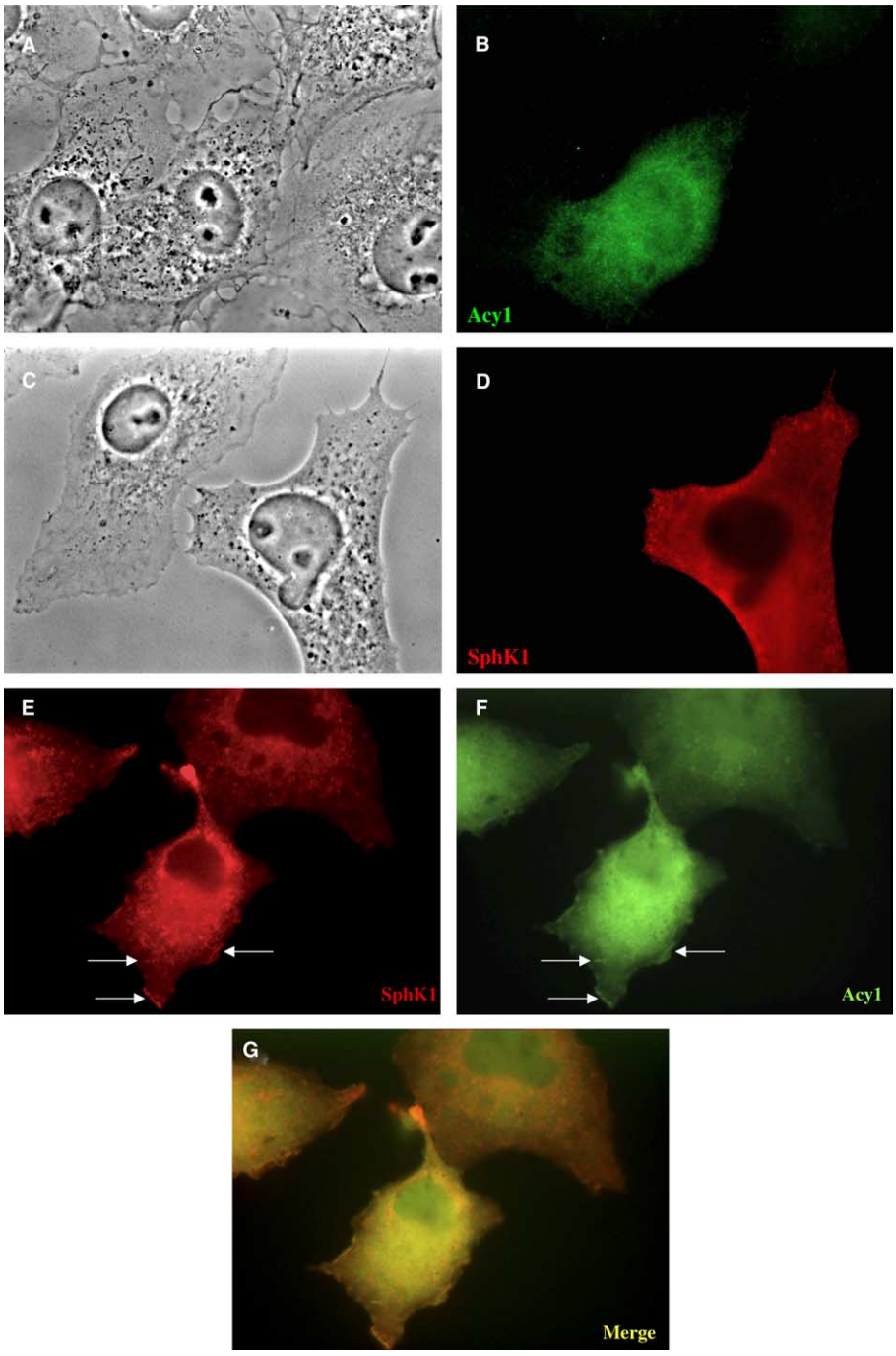

Fig. 3. Acy1 alters the intracellular distribution of SphK1. Cos7 cells were transfected with V5-Acy1 (A,B) or myc-SphK1 (C,D) or both (E–G) and fixed 48 h later. Cells were then incubated with anti-myc and anti-V5 antibodies and stained with Texas red anti-mouse IgG and FITC anti-rabbit IgG. Phase (A,C) and fluorescent (B,D,E–G) images were obtained with a Nikon TE-200 using a CoolSnap camera driven by MetaMorph software. Panel G shows the superimposed merged pictures, yellow color represents co-localization of the two proteins. Arrows indicate long tubular structures observed only when proteins were co-transfected.

negative inhibitor of SphK1. We suspect that overexpression of CT-Acy1 blocks the ability of SphK1 to interact with endogenous, active Acy1. This would block the pro-growth and anti-apoptotic effects of SphK1 if the aminoacylase activity of Acy1 is required for its SphK1 regulatory effects, because CT-Acy1 is enzymatically inactive. It is also possible that the N-terminus of Acy1, missing from CT-Acy1, may have binding sites for other proteins required for the SphK1–Acy1 complex to inhibit apoptosis and promote cell growth or for its translocation to its site of action.

Because cellular levels of the bioactive sphingolipid mediator S1P are low and tightly regulated, it is not surprising that cells have evolved many mechanisms to control the activity of SphK1, the critical enzyme responsible for formation of S1P, as suggested by the discovery of a plethora of SphK1-interacting proteins [17–20]. Most of them, including Acy1, have in

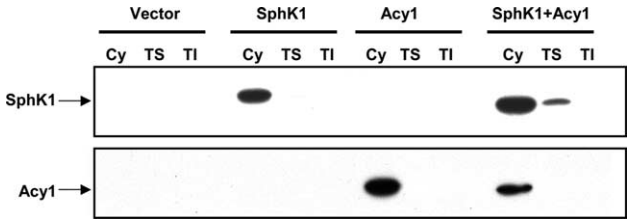

Fig. 4. Acy1 translocates SphK1 from the cytosol to the Triton-soluble (TS) membrane fraction. HEK 293 cells were transfected with SphK1, Acy1, or both. After 48 h, cells were harvested and lysed by freeze-thawing. The lysates were centrifuged at $100\,000 \times g$ to generate cytosol (Cy) and pellet fractions. 1% Triton X-100 was added to the pellet fractions and after centrifugation at $100\,000 \times g$, equal amounts of the Triton X-100-insoluble (TI) and TS fractions were separated on 10% SDS–PAGE, transblotted to nitrocellulose, and probed with antibodies to myc (SphK1) and V5 (Acy1) epitopes.

common the ability to reduce SphK1 enzymatic activity and affect its cellular localization, directing it from a diffuse cytoplasmic expression to specific membranes where S1P production can then be spatially and temporally regulated to influence both intracellular and extracellular signaling pathways.

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