

# A rapid fluorescence assay for sphingosine-1-phosphate lyase enzyme activity

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**Abstract** Sphingosine-1-phosphate (S1P) lyase (SPL) catalyzes the conversion of S1P to ethanolamine phosphate and hexadecenal. This enzyme plays diverse roles in physiology and disease and, thus, may be useful as a disease marker and/or drug target. Unfortunately, the radioisotope-based assay currently used to quantify SPL activity is suboptimal. We have devised an assay using a commercially available  $\omega$ (7-nitro-2-1,3-benzoxadiazol-4-yl)-D-erythro (NBD)-labeled fluorescent substrate. Alternatively, we provide a method for synthesis of the substrate from NBD-sphingosine. Enzyme activity is determined by following the formation of NBD-aldehyde product, which is isolated from unreacted substrate by lipid extraction and quantified after separation by HPLC using a C18 column. A fluorescent NBD-C18-sphingosine internal standard is used to control for extraction efficiency. The reaction is linear over 20 min and total protein concentrations of 20–200 mg/l. The sensitivity of the fluorescence assay is comparable to or better than that of the radioactive assay, and SPL levels as low as 8 pmol/mg/min were readily detected. Semicarbazide, a nonspecific SPL inhibitor, reduced SPL activity in vitro by ~70% using both standard and fluorescence methods. Product inhibition was not observed using ethanolamine phosphate and a commercially available source of hexadecenal. This method is suitable for quantifying SPL activity in a variety of cell and tissue sources.—Bandhuvula, P., H. Fyrst, and J. D. Saba. A rapid fluorescence assay for sphingosine-1-phosphate lyase enzyme activity. *J. Lipid Res.* 2007. 48: 2769–2778.

**Supplementary key words** high-performance liquid chromatography •  $\omega$ (7-nitro-2-1,3-benzoxadiazol-4-yl)-D-erythro-sphingosine • sphingosine-1-phosphate • dihydrosphingosine-1-phosphate

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid that regulates cell survival, migration, angiogenesis, and lymphocyte trafficking (1–7). Sphingosine-1-phosphate lyase (SPL) catalyzes the cleavage of S1P and other long-chain base phosphates (LCBPs) at the C<sub>2</sub>-C<sub>3</sub> bond, yielding ethanolamine phosphate and a long-chain aldehyde (8). The enzyme is highly conserved throughout evolution, and functional homologs have been identified in yeast, insects,

nematodes, slime mold, and vertebrates (9–12). SPL function contributes to development, survival, stress responses, tissue integrity, and pathogen infectivity. Furthermore, SPL expression is downregulated in intestinal tumorigenesis (13–15), and pharmacologic inhibition of SPL activity blocks lymphocyte trafficking (7). These findings demonstrate the diverse roles that SPL plays in biology and raise the possibility that it may be a useful target for antibiotic, immunomodulatory, and anticancer therapy. Unfortunately, the assay currently used to quantify SPL activity is cumbersome and expensive and uses a radioactive substrate for which there is only one commercial source, which we have found to be impure, leading to high background and low sensitivity (16). Accurate determination of SPL activity using the standard assay necessitates exposing an autoradiogram for 2 weeks to locate and scrape the tritium-labeled reaction products from a thin-layer chromatography plate. Additional time is often required to purify commercially available substrate. To avoid these difficulties, we have devised an SPL enzyme assay using an  $\omega$ (7-nitro-2-1,3-benzoxadiazol-4-yl)-D-erythro (NBD)-labeled fluorescent substrate that is effectively catabolized by SPL, yielding a fluorescent product that can be separated from substrate by lipid extraction and detected by separation with HPLC using a C18 column in a reaction that can be completed in 1 day.

## EXPERIMENTAL PROCEDURES

### Materials

NBD-sphingosine and NBD-S1P were from Avanti Polar Lipids (Alabaster, AL). [4,5-<sup>3</sup>H]dihydrosphingosine-1-phosphate (DHS1P) and [1-<sup>3</sup>H]sphingosine were from American Radio-labeled Chemicals, Inc. (St. Louis, MO). C18-DHS1P and C18-

Abbreviations: CLAP, chymostatin, leupeptin, antipain, and pepstatin A; DHS1P, dihydrosphingosine-1-phosphate; 2,4-DNPH, 2,4-dinitrophenylhydrazine; GFP, green fluorescent protein; LCBP, long-chain base phosphate; NBD,  $\omega$ (7-nitro-2-1,3-benzoxadiazol-4-yl)-D-erythro; S1P, sphingosine-1-phosphate; Sphk1, sphingosine kinase 1; SPL, sphingosine-1-phosphate lyase; TBAP, tetrabutylammonium-dihydrogen phosphate.

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DHS were from Matreya, LLC (Pleasant Gap, PA). FTY720 was a kind gift from Volker Brinkmann (Novartis).

### Cell lines and preparation of cell extracts

Human embryonic kidney cells (HEK293), DLD1 colon cancer cells, and murine 3T3-L1 cells were obtained from the American Type Culture Collection (Rockville, MD) and were cultured in Dulbecco's modified Eagle's medium (Tissue Culture Facility, University of California, San Francisco) containing 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). *Drosophila mbn2* cells were obtained from the *Drosophila* Stock Center (Bloomington, IN) and were grown in Schneider 2 medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). *Saccharomyces cerevisiae* wild type (JSK406), *dpl1* deletion mutant (JSK385), and *DPL1* overexpression (JSK460) strains were grown under standard conditions as described previously (17). Human SPL and murine sphingosine kinase 1 (Sphk1) were expressed in HEK293 cells using an adenoviral expression system essentially as described (15). Cells were infected with adenovirus expressing a control green fluorescent protein (GFP) marker (Ad-GFP) or human SPL and a GFP marker (Ad-SPL) at a multiplicity of infection of 100. Infection efficiency was determined to be >90% by quantifying GFP-positive cells using fluorescence microscopy. Cells were lysed by glass bead disruption for 15 min in SPL extraction buffer [0.05 M potassium phosphate buffer, pH 7.2, 2 mM EDTA, 0.2 mM pyridoxal 5' phosphate, 2 mM 2-mercaptoethanol, 11% glycerol, 1 mM PMSF, and 25 µg/ml chymostatin, leupeptin, antipain, and pepstatin A (CLAP)]. Extracts from *S. cerevisiae* and *Leishmania major* were prepared by glass bead disruption in SPL extraction buffer at 4°C. Whole cell extracts were used, with the exception of *Leishmania*, in which the microsomal fraction was isolated and used for SPL assays.

### Preparation of tissue extracts

Mouse euthanasia and tissue harvest were performed in accordance with an approved Institutional Animal Care and Use Committee protocol. Mouse organs were homogenized by tip sonication in tissue lysis buffer (5 mM MOPS, 1 mM DTT, 1 mM EDTA, 0.25 M sucrose, 10% glycerol with PMSF, and 25 µg/ml CLAP). The homogenates were subjected to centrifugation at 500 g to clarify the extracts. The supernatant was used for SPL activity assays.

### Evaluation and purification of [4,5-<sup>3</sup>H]DHS1P

The purity of commercially purchased substrate was evaluated by mixing 100–200 pmol of cold DHS1P with an aliquot of [4,5-<sup>3</sup>H]DHS1P, derivatized with *ortho*-phthaldialdehyde (17) and separated by isocratic elution on a 4.6 × 75 mm Luna C18 column (Phenomenex, Torrance, CA), with the mobile phase methanol-water-1 M tetrabutylammonium-dihydrogen phosphate (TBAP; 84.1:15:0.9). The flow rate was 1 ml/min. The fluorescent *ortho*-phthaldialdehyde compounds were detected using an SP 8410 fluorescence detector (Spectra-Physics, Mountain View, CA) (18). The peak corresponding to DHS1P was collected and analyzed by scintillation counting. The radioactive substrate was purified before use as follows. The substrate was purified by a two-phase extraction in which 1 volume of aqueous phase containing a final concentration of 1.5 M ammonium hydroxide was mixed with 2 volumes of chloroform-methanol (2:1). The upper aqueous phase containing the substrate was recovered and acidified by adding acetic acid at a final concentration of 3.0 M. Another two-phase separation was obtained by adding 2 volumes of chloroform-methanol (2:1), and the substrate was isolated in the organic phase. An aliquot of the

purified substrate was also evaluated by running TLC on silica gel 60 plates (EM Sciences) in butanol-acetic acid-water (3:1:1). Each TLC spot was scraped, and substrate purity was determined as the percentage of total recovered counts that were accounted for by the DHS1P spot at Rf = 0.3. Using these methods, substrate was purified to 80% or greater and used for all radioactive assays.

### Radioactive SPL assay

Standard radiometric SPL assays were performed using [4,5-<sup>3</sup>H]DHS1P as substrate, essentially as described (16) but with minor modifications. For the assay, 5 nmol of [<sup>3</sup>H]DHS1P (specific activity of 0.025 µCi/nmol) was dissolved in 175 µl of SPL reaction buffer (0.6 mM EDTA, 0.4 mM pyridoxal 5'-phosphate, 3 mM DTT, 70 mM sucrose, 36 mM potassium phosphate buffer, and 36 mM NaF) containing 0.08% Triton X-100 by sonication. Cell extracts containing 25–50 µg of total protein in a volume of 50 µl were added to start the reaction, which proceeded at 37°C for 15 min or longer, as indicated. In all standard assays, the final concentration of DHS1P was kept at 20 µM to ensure maximum reaction rates. Furthermore, Triton X-100 was kept at 1 mM to ensure the formation of micellar structures. Boiled cell extracts were used as blanks. The products of the reaction were extracted and separated using TLC as described (16). A correction for loss of product through the extraction and TLC was introduced into the standard assay by determining the percentage recovery of radioactive [4,5-<sup>3</sup>H]DHS1P after extraction and TLC in a mock assay to be 33%.

### Fluorescent SPL assay

The assay was performed as above with the following changes: 5 nmol of fluorescent NBD-S1P substrate was used in place of the radioactive DHS1P substrate. Instead of separating the product by TLC, the organic phase containing the product was dried down in a vacuum dryer, resuspended in methanol, and injected onto the HPLC system. As an internal standard, 200 pmol of NBD-C18-sphingosine was added after the enzyme reaction was complete and before lipid extraction. NBD-labeled compounds were separated by HPLC on a C18 column at a flow rate of 1 ml/min. Solvents used for separation were solvent A (water) and solvent B (methanol/5 mM acetic acid in water/1 M TBAP, 95:4:1). The gradient used was 0–2 min at 60% solvent B, 2–5 min from 60% to 100% solvent B, and 5–9 min at 100% solvent B. The fluorescent NBD compounds were detected as described above and quantified by integrating peaks and comparing the signals with NBD-C18-sphingosine. During assay development, products were analyzed by TLC in comparison with commercial standards and radioactive assay products. Product Rf values were determined by localizing the fluorescent spots using an Alpha Innotech MultiImage Light Cabinet.

### Aldehyde labeling

The aldehyde product from the SPL reaction was identified after 2,4-dinitrophenylhydrazine (2,4-DNPH) labeling. The SPL product mixture was dissolved in 20 µl of methanol, and the labeling was started by adding 20 µl of 5 mM 2,4-DNPH in methanol containing 2 M HCl. The reaction was allowed to carry on for 1 h in the dark. After incubation, the reaction mixture was injected onto the HPLC system and compounds were separated and detected as described above.

### Alkaline phosphatase assays

To quantitate the conversion of NBD-S1P to NBD-sphingosine, 0.025 units of alkaline phosphatase purified from bovine intestinal mucosa (Sigma) was added to the fluorescence assay along

with NBD-S1P substrate under the conditions described above, except that NBD-sphingosine was not used as an internal standard. Reaction proceeded for 15 min, and NBD-sphingosine was quantitated by HPLC. To confirm the activity of the alkaline phosphatase under favorable conditions for this enzyme, 10  $\mu$ M NBD-S1P was solubilized in 0.08% Triton X-100 and 175  $\mu$ l of reaction buffer (1 M diethanolamine and 0.5 mM MgCl<sub>2</sub>, pH 9.8) was added. The reaction was allowed to proceed at 37°C for 15 min and was terminated by the addition of 300  $\mu$ l of 1% perchloric acid. The product was isolated and analyzed by HPLC, as described above.

### Inhibitor assays

Studies with semicarbazide and FTY720 were performed by adding inhibitor to reaction buffer, followed by the addition of extract to initiate the reaction. For some radioactive and fluorescence assays using FTY720, BSA was used in place of Triton X-100 in the reaction buffer. For these experiments, 175  $\mu$ l of the reaction buffer containing 6.4  $\mu$ M fat-free BSA and 30  $\mu$ M FTY720 was added slowly to glass tubes containing dried NBD-labeled or <sup>3</sup>H-labeled substrates while vortexing and sonicating in a bath sonicator for 30 s. The reaction was started by the addition of 50  $\mu$ l of Ad-SPL whole cell extract containing 25–50  $\mu$ g of protein.

### Preparation of substrate from NBD-sphingosine

HEK293 cells expressing murine Sphk1 were generated using an adenoviral expression system. Infection occurred at nearly 100% efficiency as determined by GFP fluorescence, and the cells exhibited  $\sim$ 2 nmol/mg/min sphingosine kinase activity, determined using a standard assay based on the phosphorylation of a [1-<sup>3</sup>H]sphingosine substrate (18). Fresh whole cell extracts were prepared by glass bead disruption in sphingosine kinase extraction buffer (20 mM Tris-HCl buffer, pH 7.4, 1 mM EDTA, 0.5 mM 4-deoxypyridoxine, 15 mM NaF, 20% glycerol, and 25  $\mu$ g/ml CLAP). To generate the sphingosine kinase product, 100 nmol of NBD-sphingosine was mixed with C16-lysophosphatidylcholine (1:9, mol/mol) and dried down using a vacuum dryer. The substrate mixture was resuspended in 500  $\mu$ l of 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5 mM 4-deoxypyridoxine, 15 mM NaF, 1 mM 2-mercaptoethanol, and 25  $\mu$ g/ml CLAP by tip sonication for 30 s followed by the addition of 1 ml of reaction buffer containing 100 mM MOPS, pH 7.2, 5 mM 2-mercaptoethanol, 15 mM magnesium chloride, and cell extract containing 500  $\mu$ g of protein. The sphingosine kinase reaction was started by the addition of 250  $\mu$ l of 10 mM ATP and allowed to proceed for 1 h. The reaction was stopped by adding ammonium hydroxide to a final concentration of 1.5 M, and NBD-S1P was isolated and purified by two-phase extraction as described above. The purity of the NBD-S1P was verified by HPLC and found to be >90%.

### Protein determination

Total protein determination was performed by the Bradford method (19).

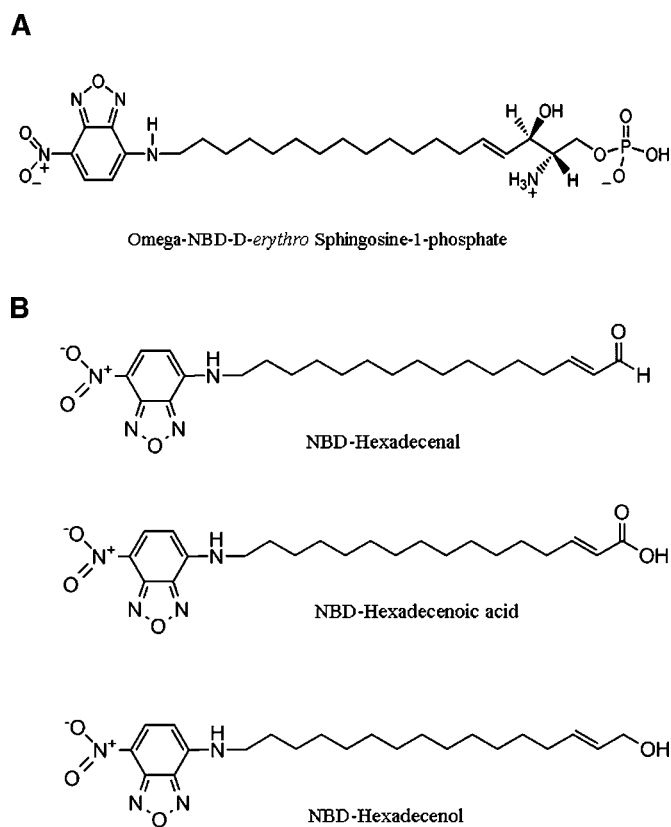
## RESULTS

### Formation of NBD-labeled products from NBD-S1P by SPL

SPL is able to catalyze the cleavage of multiple LCBPs including S1P, DHS1P, phytosphingosine-1-phosphate, and LCBPs of multiple chain lengths (8). The reaction is thought to involve the formation of a Schiff base between the LCBP substrate and the cofactor pyridoxal 5'-phosphate, which itself is loosely associated with the enzyme.

The two products of the reaction are ethanolamine phosphate and hexadecenal, but the latter molecule is unstable and converts *in vitro* to the corresponding fatty acid and alcohol. Thus, in the standard SPL assay, all three products are usually scraped from the TLC plate and counted together. The efficacy of the radioactive assay depends upon the purity and specific activity of the [4,5-<sup>3</sup>H]DHS1P substrate. To determine the purity of the radioactive substrate, we quantitated the total amount of radioactivity in individual substrate lots and correlated this with the amount of radioactivity recovered in fractions corresponding to the DHS1P peak observed by HPLC using isocratic elution with methanol/water/1 M TBAP (84:15:1) at a flow rate of 1 ml/min. Using this method, we found the purity of the commercially available substrate to be highly variable between different lots, with a maximum purity of <50%, thereby necessitating further purification. After purification by two-phase extraction as described in Experimental Procedures, we were able to obtain a substrate with a purity of at least 80%. However, our purified substrate still showed a 10–15% variation in purity depending on the substrate lot in hand, thereby complicating comparisons of specific as well as enzymatic activities.

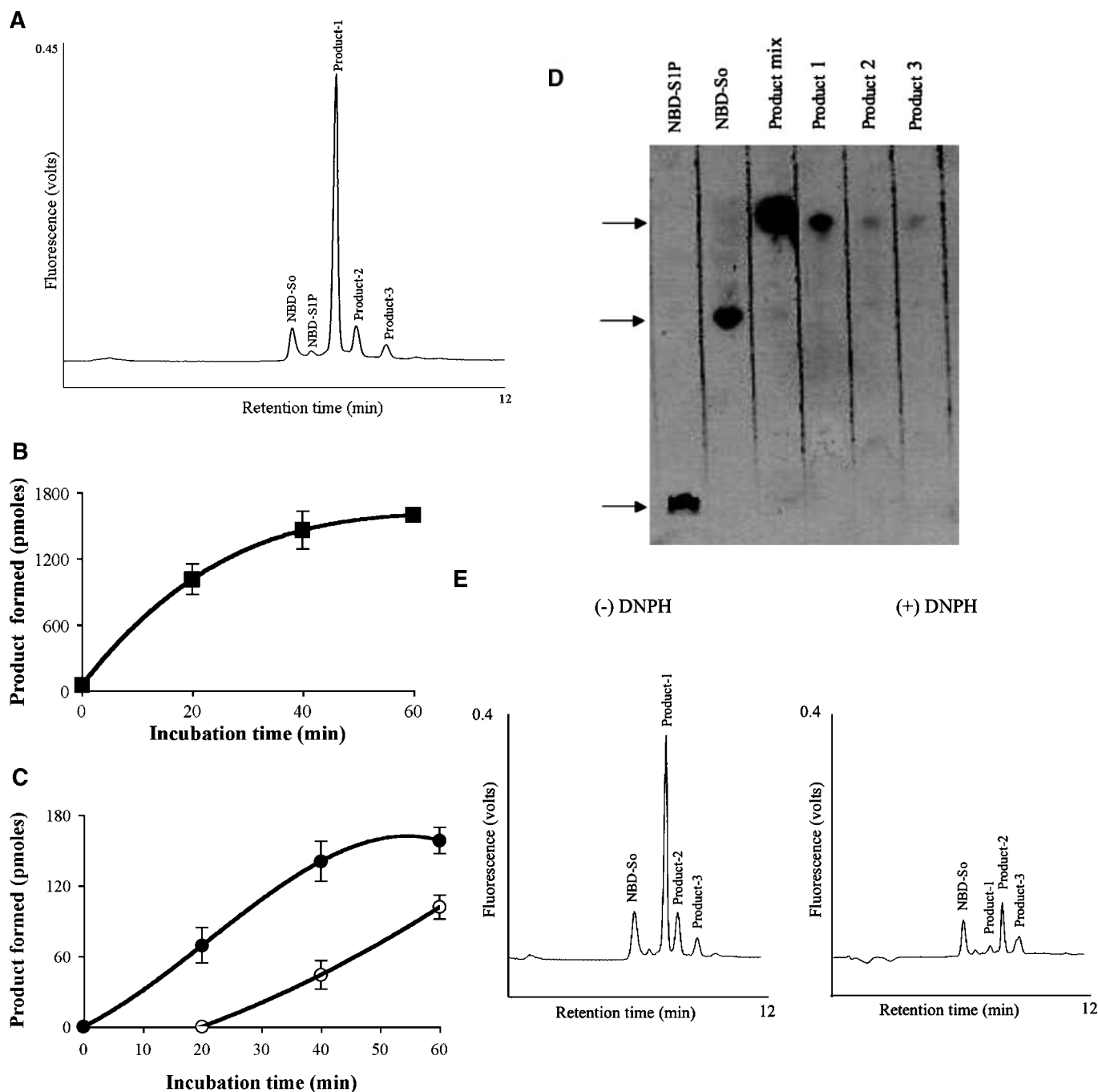
NBD-S1P is a stable compound that contains a fluorescent group attached to the terminal carbon of the long-chain base *D-erythro*-sphingosine ( $\omega$ -NBD-S1P) (Fig. 1A).



**Fig. 1.** Structures of  $\omega$ (7-nitro-2,1,3-benzoxadiazol-4-yl)-*D-erythro*-sphingosine-1-phosphate (NBD-S1P) and corresponding products of the sphingosine-1-phosphate lyase (SPL) reaction. Shown are NBD-S1P (A) and the three expected NBD-labeled reaction products (B).

We reasoned that an  $\omega$ -linked NBD group would not substantially hinder the SPL reaction and that the products retaining the NBD label (Fig. 1B) would be simple to monitor by HPLC and fluorescence detection. Therefore, to determine whether an assay for SPL activity could be developed based on the conversion of NBD-S1P to fluorescent products, NBD-S1P was incubated with cell ex-

tracts containing  $\sim 1$ – $1.5$  nmol/mg/min SPL activity by virtue of Ad-SPL. The reaction was performed and NBD compounds were extracted and separated by HPLC as described in Experimental Procedures. Using this method, one suspected product peak (peak 1) was identified at 7.4 min and two smaller peaks (peaks 2 and 3) were identified at 8.0 and 8.8 min (Fig. 2A). These peaks were



**Fig. 2.** Identification of NBD products. The SPL reaction was carried out with 20  $\mu$ M NBD-S1P substrate and incubated for 30 min with 25  $\mu$ g of Ad-SPL protein. Lipids were extracted, and NBD compounds were separated by HPLC. A: Chromatogram showing the separation of NBD-sphingosine, NBD-S1P, and NBD products 1, 2, and 3. B: Time-dependent formation of product 1. C: Time-dependent formation of product 2 (closed circles) and product 3 (open circles). D: TLC analysis of NBD-labeled compounds, including SPL reaction products before and after HPLC separation. R<sub>f</sub> values are shown in Table 1. E: Chromatogram showing NBD products 1, 2, and 3 before and after labeling with 2,4-dinitrophenylhydrazine (2,4-DNPH). Each data point in B and C represents the mean  $\pm$  SD of at least three independent experiments.

easily distinguishable from that of NBD-S1P, which had a retention time of 6.8 min, and NBD-sphingosine, which had a retention time of 6.2 min (Fig. 2A). These findings indicate that one major product peak was observed in the fluorescence assay, with two additional peaks constituting a significantly smaller portion of the total product recovered.

### Verification of SPL products

To verify that all three HPLC peaks were indeed SPL reaction products, we determined the increases in product peak 1 (Fig. 2B) and peaks 2 and 3 (Fig. 2C) as functions of time. As shown in the figure, all three products increased steadily as the reaction proceeded. These data strongly suggest that the peaks represent SPL reaction products. Further identification of the NBD-labeled SPL products was performed by applying several different techniques. Total product isolated after lipid extraction as well as HPLC fractions containing individual SPL products were subjected to TLC separation. Product spots were compared side by side with an 11-*cis*-hexadecanal standard, a palmitic acid standard, and metabolites of the standard SPL reaction (using DHS1P substrate). The three NBD-labeled products were identified by fluorescence detection using a digital scanner, whereas nonfluorescent standards and SPL metabolites were identified using iodine staining. As shown in Fig. 2D, NBD-labeled SPL products 1, 2, and 3 were not distinguishable from one another by TLC and demonstrated Rf values between 0.8 and 0.82. By comparison, we found the Rf values of the standard SPL reaction products as well as 11-*cis*-hexadecanal to be similar (Table 1). As the  $\omega$ -linked NBD group had no effect on the Rf value of C18-sphingosine in our TLC system (data not shown), we conclude that product peaks 1–3 originate from the SPL reaction.

Our findings are slightly different from the published values for SPL metabolites (0.87 for C16 aldehyde, 0.84 for C16 fatty alcohol, and 0.80 for C16 fatty acid) (16), most likely as a result of the different composition of our TLC plates. Although the substrate and product peaks were readily distinguished in our assay system, complex mixtures could potentially present more difficulty in distinguishing these peaks. When a longer (250  $\times$  4.6 mm Luna C18) column was used, Rf values of 25 min for NBD-S1P, 27.5 min for product 1, and 32.6 min for product 2 were obtained. Thus, when analyzing SPL activity in complex mixtures, a longer column can help to improve the resolution of substrate and product peaks. The compound 2,4-DNPH, which reacts with carbonyl groups,

was used to identify the aldehyde product from the SPL reaction. HPLC separation of the SPL product mixture after labeling with 2,4-DNPH was then performed. As shown in Fig. 2E, incubation of the SPL product mixture with 2,4-DNPH reduced the signal from product peak 1 to <5% compared with the unlabeled SPL product mixture. In contrast, the signals from product peaks 2 and 3 were not affected by incubation with 2,4-DNPH. These data demonstrate that peak 1 represents the aldehyde product. Moreover, the HPLC separation indicates that the aldehyde is the major product obtained under the assay conditions used, in agreement with published data (16). Attempts to further confirm the identity of individual HPLC peaks using ESI mass spectrometry were not successful, because of the instability of the NBD label in the ion source and impurities originating from the SPL reaction.

### Phosphatases are not active under SPL assay conditions

To avoid competition for substrate between SPL and SIP phosphatases potentially present in biological samples, NaF is added to the reaction. The reaction conditions of the SPL assay are not favorable to most phosphatases, because of the presence of high inorganic phosphate, EDTA, and (for alkaline phosphatases) neutral pH. However, to rule out the possibility that phosphatases may contribute to substrate degradation in our assay system, the assay was performed in the presence of high concentrations of pure mammalian alkaline phosphatase. Under optimal conditions for alkaline phosphatase, NBD-S1P was readily converted to NBD-sphingosine (20% conversion in 15 min), demonstrating robust enzyme activity. In contrast, under SPL assay conditions, no conversion of NBD-S1P to NBD-sphingosine was detected (data not shown). Similar results were obtained using murine intestinal extract, which contains a high content of alkaline phosphatase (data not shown). These results demonstrate that phosphatases do not interfere with the SPL reaction in our assay system.

### Quantitation of NBD-labeled SPL products

To compare the SPL activity results obtained with the fluorescent and radioactive substrate, protein extracts were incubated with either NBD-S1P or [4,5-<sup>3</sup>H]DHS1P under identical conditions, followed by separation and detection. To accurately quantitate the amount of NBD-labeled products formed in the fluorescence assay, NBD-sphingosine was used as an internal standard. By HPLC analysis, recoveries of 62.7% for the NBD-sphingosine standard and 67.3% for the NBD-labeled products were

TABLE 1. LC analysis of sphingosine-1-phosphate lyase reaction products and related lipids

Parameter	Palmitic Acid	11- <i>cis</i> -Hexadecenal	<sup>3</sup> H-Labeled Product Mixture	NBD-Labeled Product Mixture	NBD Product 1	NBD Product 2	NBD Product 3
Rf value	0.76	0.81	0.79–0.81	0.80–0.82	0.81	0.82	0.82

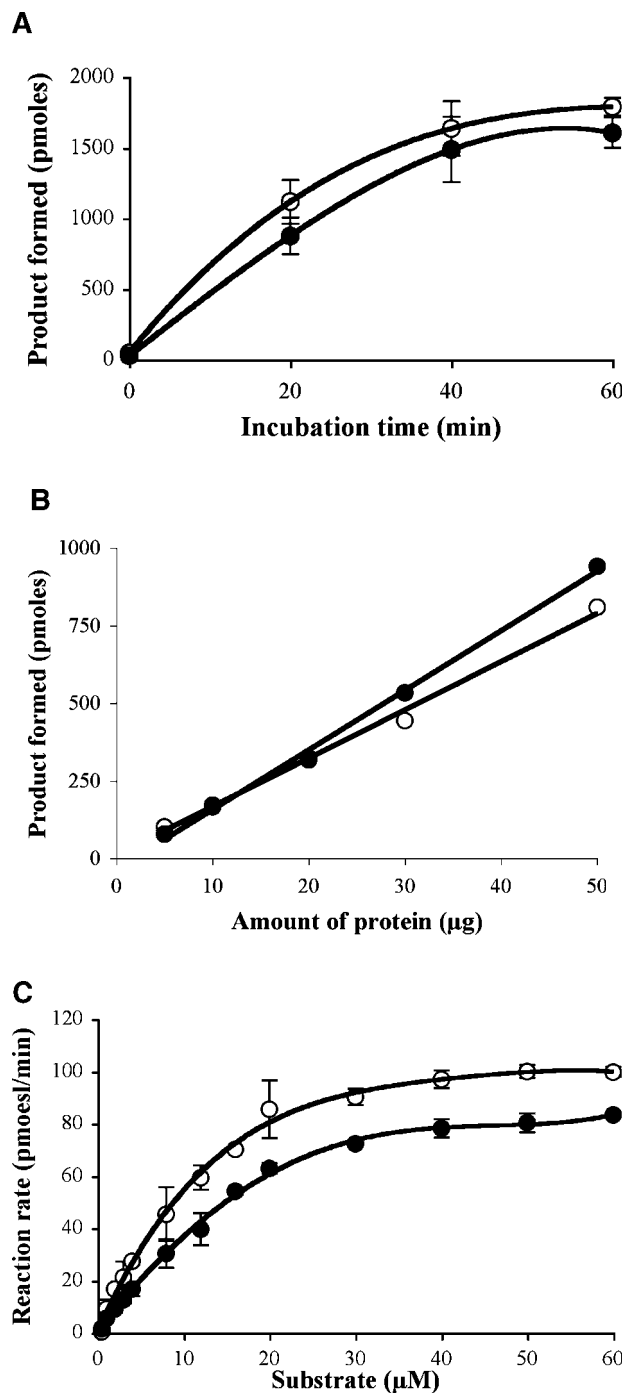
NBD,  $\omega$ (7-nitro-2-*l*,3-benzoxadiazol-4-yl)-*D*-erythro. Lipids were separated on silica gel 60 plates with the mobile phase butanol-acetic acid-water (3:1:1). Values represent averages of at least three independent runs.

obtained. Because the recoveries of NBD-sphingosine and all of the NBD-labeled SPL reaction products were similar throughout the extraction procedure, NBD-sphingosine was considered an appropriate substitute for NBD-labeled products, which are not commercially available. Similarly, a correction factor was used in the standard assay based on the recovery of radioactive [4,5-<sup>3</sup>H]DHS1P after extraction and TLC, as described in Experimental Procedures. Using the internal standard and the correction factor, the amount of NBD product formed could be calculated by combining the area of product peaks 1, 2, and 3 and the amount of radiolabeled product formed by scintillation counting of the TLC area corresponding to SPL product standards. Using this method of quantitation, product formation was quantitated as a function of time.

As shown in Fig. 3A, the two reactions gave similar results up to 60 min of incubation. In both cases, the reaction appeared nonlinear after 20 min. Commercially available NBD-S1P and NBD-S1P synthesized by in vitro phosphorylation of NBD-sphingosine using murine Sphk1 were effective substrates (data not shown). To assess the effect of protein content on assay results, both radioactive and fluorescence assays were performed simultaneously with varied amounts of total protein in the reaction. Both standard and fluorescence assays yielded similar results and demonstrated an increase in the accumulation of the product corresponding to an increase in total protein (Fig. 3B). To determine the SPL activity at different concentrations of NBD-S1P and DHS1P substrate, the concentration of Triton X-100 was maintained at 1 mM, significantly above the critical micelle concentration of 0.24 mM, whereas the substrate concentration was varied from 1 to 60  $\mu$ M. The reaction was stopped at 15 min to maintain the reaction in the linear range with respect to time. As shown in Fig. 3C, the enzymatic reaction proceeded at similar rates with both substrates. The reaction was linear up to 12  $\mu$ M substrate. For NBD-S1P, a  $K_m$  of 14.6  $\mu$ M was determined, and radiolabeled DHS1P resulted in a  $K_m$  of 20.1  $\mu$ M, indicating close correlation between the assay systems, consistent with previous findings for the standard assay (16). Repeat analyses using cell extracts with lower enzyme activities resulted in <10% conversion of substrate, and calculated  $K_m$  values were within a similar range (7  $\mu$ M for NBD-S1P and 10  $\mu$ M for DHS1P).

### Stability of the products

To determine the stability of the NBD-labeled products of the reaction, products from one assay were extracted and divided into two aliquots. One of these aliquots was run on HPLC the same day, whereas the other aliquot was stored at  $-20^\circ\text{C}$  for 1 week, followed by HPLC analysis. Less than a 10% difference was observed in the individual peak values as well as in total SPL activity between the two aliquots (data not shown). These results indicate that the assay can be completed in 1 day or performed in an interrupted manner without loss of product for up to 1 week.



**Fig. 3.** Quantitation of SPL reaction products. The SPL reaction was carried out with either NBD-S1P substrate (open circles) or [4,5-<sup>3</sup>H]dihydro sphingosine-1-phosphate (DHS1P) substrate (closed circles). **A:** Time-dependent formation of SPL products with 20  $\mu$ M substrate and 25  $\mu$ g of Ad-SPL total protein. Each data point represents the mean  $\pm$  SD of at least three independent experiments. **B:** Protein-dependent formation of SPL products with mouse thymus extract and 20  $\mu$ M substrate. Each data point represents the mean of two independent experiments. **C:** Substrate-dependent formation of SPL products with 15  $\mu$ g of Ad-SPL total protein for 15 min. Each data point represents the mean  $\pm$  SD of at least three independent experiments.

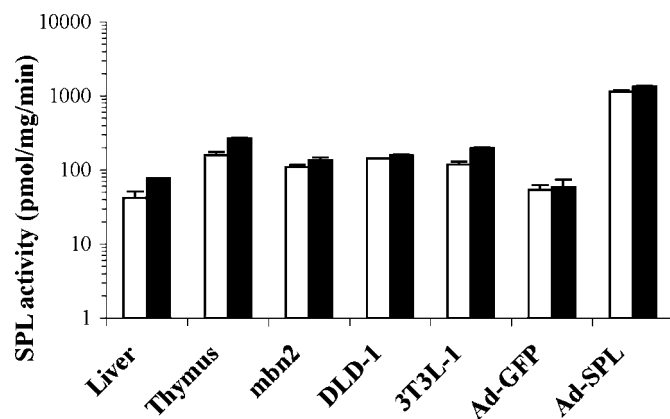


Fig. 4. SPL activity in different cell extracts. The SPL reaction was carried out with 25  $\mu$ g of total protein and 20  $\mu$ M NBD-S1P substrate (open bars) or [4,5-<sup>3</sup>H]DHSIP substrate (closed bars). Each data point represents the mean  $\pm$  SD of at least three independent experiments.

#### The fluorescent SPL assay is applicable for a wide range of biological specimens

To examine the applicability of the fluorescence assay to mammalian tissues and cells and its potential use in the

analysis of clinical samples, the fluorescence assay was performed on a range of mammalian cells and tissues, including HEK293 cells expressing high levels of human SPL by virtue of an adenoviral expression system (Ad-SPL), HEK293 cells containing a control adenoviral vector (Ad-GFP), DLD1 colon cancer cells, murine thymus and liver, and murine 3T3-L1 cells. As shown in Fig. 4, in each case there was close agreement between the fluorescence and radioactive assays in the quantitation of SPL activity. SPL activity determinations in extracts of the *Drosophila* mbn2 hemocyte cell line using both assay systems were also similar (Fig. 4). These data indicate that the fluorescence assay is applicable over a wide range of SPL activities and for the measurement of SPL activity in cell lines and tissues of various species.

To determine whether the fluorescence assay can be used to verify the presence or absence of SPL activity in genetically modified organisms, yeast extracts from wild-type strains and strains in which the endogenous *S. cerevisiae* SPL gene *DPL1* has been deleted or over-expressed were analyzed for SPL activity using the new assay system. As shown in Fig. 5A, B, SPL activity results correlated well with the expected biochemical phenotype

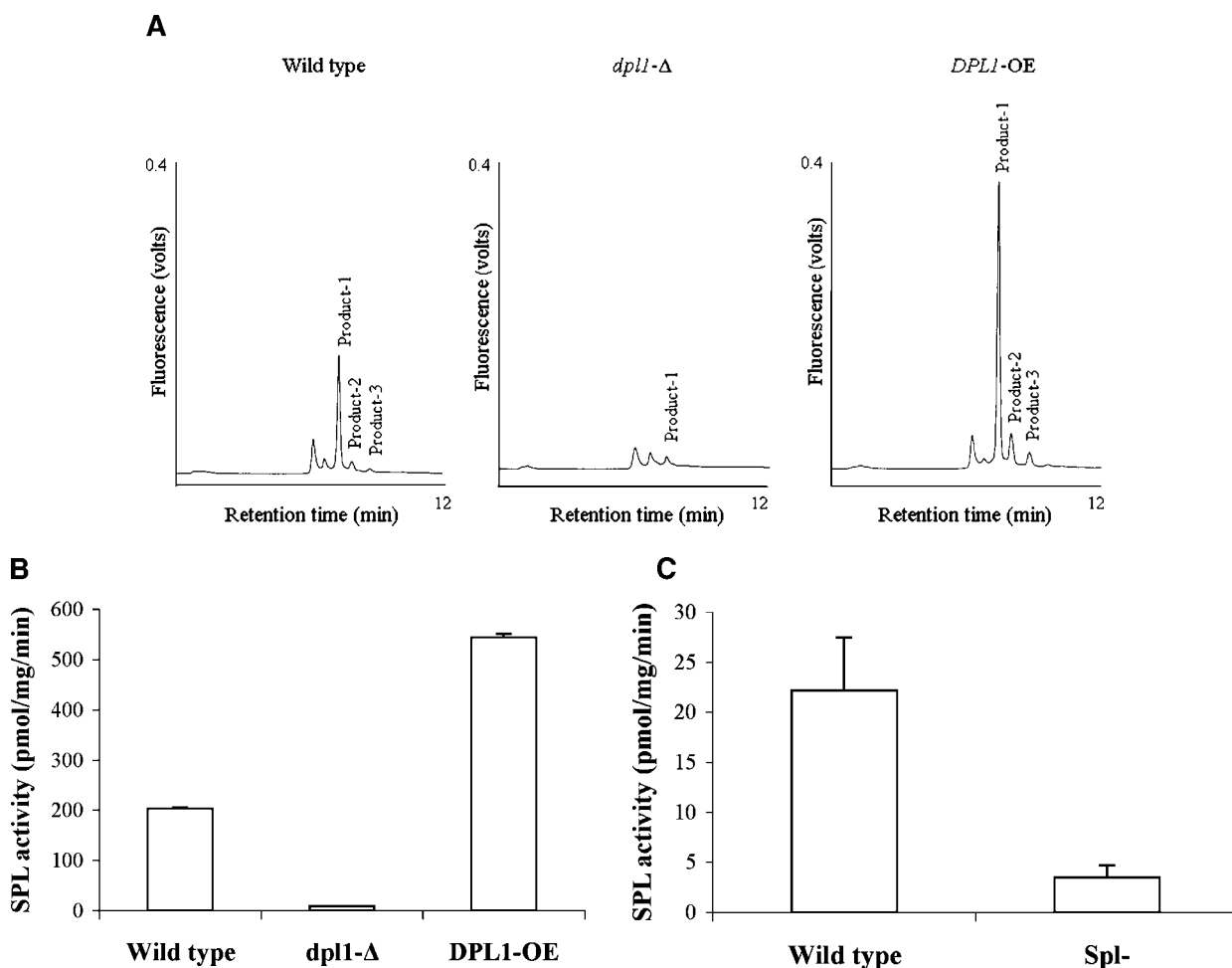


Fig. 5. SPL activity in genetically modified organisms. The SPL reaction was carried out for 30 min with 50  $\mu$ g of total protein and 20  $\mu$ M NBD-S1P substrate. A: Chromatogram of a yeast wild-type strain, a yeast SPL knockout strain (*dpl1-Δ*), and a yeast SPL-overexpressing strain (*DPL1-OE*). B: Activity in the wild type, *dpl1-Δ*, and *DPL1-OE*. C: Activity in *L. major* wild type and SPL knockout (*Spl-*). Each data point in B and C represents the mean  $\pm$  SD of at least three independent experiments.

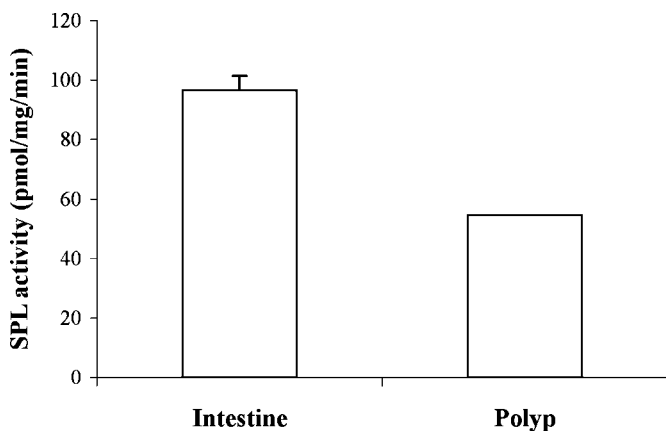
of strains with *DPL1* wild-type, null, and overexpression genotypes. Similarly, SPL activity observed in microsomal fractions of *L. major* wild-type and knockout strains were consistent with genotype, as shown in Fig. 5C.

We recently demonstrated that SPL expression and activity are downregulated in adenomas of the *Apc<sup>Min/+</sup>* mouse model of colon cancer (15). To confirm this finding, extracts from intestinal polyps and adjacent intestinal tissue devoid of macroscopic polyps were obtained from a 100 day old *Apc<sup>Min/+</sup>* mouse with florid intestinal polyposis and analyzed for SPL activity using the fluorescence assay. As shown in Fig. 6, results obtained from the fluorescence assay confirm our previously published data indicating that SPL activity is reduced in intestinal neoplasms (8, 20).

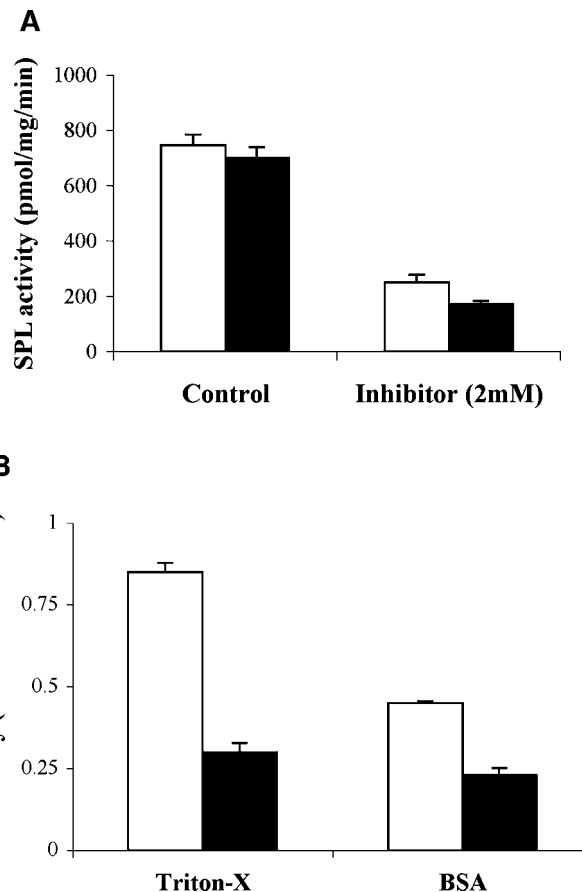
#### Fluorescence assay using SPL inhibitors

Finally, it was important to determine whether the fluorescent SPL assay may be used to test or screen for small molecule SPL inhibitors. Toward that end, fluorescent and radioactive SPL assays were performed on Ad-SPL extracts preincubated with a known SPL inhibitor, semicarbazide. In both assay systems, semicarbazide inhibited enzyme activity by ~70%, as shown in Fig. 7A. In addition, SPL assays were conducted in the presence or absence of 30  $\mu$ M of the immunomodulatory drug and sphingosine analog FTY720, which we recently established to be a modest inhibitor of SPL (21). As expected, FTY720 inhibited the reaction by 70% using the radioactive assay. In contrast, when NBD-S1P was used as the substrate, FTY720 inhibited product formation by only ~20%.

It has been reported that the kinetics of SPL are affected by the presence of Triton X-100 (16). Therefore, we considered the possibility that the structure of the substrate Triton X-100 micelles could also affect the presentation of the inhibitor to the enzyme. To address this possibility, SPL assays were conducted in which inhibitor



**Fig. 6.** SPL activity in *Apc<sup>Min/+</sup>* mouse colon tissue. The SPL reaction was carried out for 30 min with 20  $\mu$ M NBD-S1P substrate using 50  $\mu$ g of total protein from intestinal polyps and macroscopically normal-appearing adjacent intestinal tissue. Each data point represents the mean  $\pm$  SD of at least three independent experiments.



**Fig. 7.** SPL activity inhibition by semicarbazide and FTY720. A: The SPL reaction was carried out with 20  $\mu$ M NBD-S1P substrate (open bars) or 20  $\mu$ M [4,5-<sup>3</sup>H]DHS1P substrate (closed bars) for 15 min using 25  $\mu$ g of Ad-SPL total protein in the presence or absence of 2 mM semicarbazide. B: The SPL reaction was carried out with 20  $\mu$ M NBD-S1P substrate (open bars) or 20  $\mu$ M [4,5-<sup>3</sup>H]DHS1P substrate (closed bars) using 25  $\mu$ g of Ad-SPL total protein in the presence or absence (control) of 30  $\mu$ M FTY720. Before the reaction, the substrate was mixed with either 1% Triton X-100 or bound to fat-free BSA. Each data point represents the mean  $\pm$  SD of at least three independent experiments. Results are given as percentage of control (absence of FTY720).

and substrate were bound to fat-free BSA rather than dispersed in Triton X-100 micelles. Under these reaction conditions, the fluorescence assay demonstrated 55% inhibition of SPL activity by FTY720, compared with 75% inhibition demonstrated in the radioactive assay performed on the same extract (Fig. 7). To explore the possibility of product inhibition, SPL assays were conducted in the presence or absence of varying concentrations of ethanolamine phosphate, palmitate, and a commercially available long-chain unsaturated aldehyde (11-*cis*-hexadecenal), the latter as a substitute for 2-*trans*-hexadecenal, which is not commercially available. No significant or dose-dependent inhibition of SPL activity was observed in the presence of these compounds using either the fluorescent or standard SPL assay system (data not shown). Thus, there is no evidence to suggest a feedback loop affecting SPL activity.




## DISCUSSION

The growing list of biological processes affected by SIP signaling and the reliance of so many cell types on SIP for survival under stress conditions indicate that this ubiquitous lipid metabolite is physiologically important and relevant to human disease (4, 22–25). As an important regulator of available SIP signaling pools, SPL is likely to play a role in SIP-mediated biology and pathology. SPL gene expression is essential for the proper development, reproduction, and tissue homeostasis of various organisms and has been identified as a marker for ovarian cancer, atopic dermatitis, metastatic tumors, and biliary cirrhosis and as a downstream target of platelet derived growth factor signaling (26, 27). SPL is regulated by transcriptional mechanisms (28, 29). In addition, SPL activity may be regulated by nitrosylation, phosphorylation, and other modifications of the SPL polypeptide (30). The functional relevance of each of these recent observations will require the determination of SPL enzymatic activity in corresponding normal and diseased tissues in response to SPL protein modifications and in correlation with changes in gene expression. In addition, pharmacological inhibition of SPL activity induced immunosuppression in mice, probably by increasing tissue SIP levels and diminishing the SIP gradient between tissues and the blood/lymphatic circulation (7). Thus, SPL can be considered an emerging target for immunomodulatory therapy. Known SPL inhibitors include FTY720, 2-acetyl-4-tetrahydroxybutylimidazole, pyridoxal phosphate analogs, and the ceramide desaturase inhibitor GT11, but these agents are neither potent nor specific SPL inhibitors, and the identification of better inhibitors would be desirable (7, 8, 21, 31).

To quantitate SPL activity and elucidate its role in biology and pathology, a simple assay for determining enzymatic activity is important. In that regard, we have found the standard assay using a radioactive DHSIP substrate to have several shortcomings. The purity of the commercially available substrate is highly variable and always <50%, necessitating further purification before use. Whereas the standard assay requires a radioactive substrate, is cumbersome, and takes 2 weeks to complete, we have developed an assay that relies instead on a fluorescent NBD-labeled SIP substrate and follows the formation of product by HPLC separation and fluorescence detection. This assay can be easily completed in 1 day and avoids the expense, biohazard, and time associated with performing the standard assay.

Our analysis of the kinetics of the enzyme reaction revealed a  $K_m$  range of 7–15  $\mu\text{M}$  for the NBD-labeled substrate and 10–20  $\mu\text{M}$  for the radioactive substrate. These results are similar to those reported previously (16). The discrepancy in kinetics may be explained by alterations in the structure of the substrate Triton X-100 micelles that affect substrate interactions with the enzyme. This scenario seems likely, considering the structure of the fluorescent NBD group, and should be taken into account when considering other  $\omega$ -linked fluorescent SPL substrates. We also observed a difference in the ability of the

compound FTY720 to inhibit SPL activity toward the NBD-labeled substrate compared with the DHSIP substrate. Again, this effect is likely to be accounted for by differences in micellar structures, because it is improved when using a substrate-delivery system containing BSA instead of Triton X-100. However, it is also conceivable that the bulky side group of the fluorescent substrate may interfere with or interact directly with the inhibitor.

In conclusion, our results indicate that NBD-SIP can be used as a substrate to measure SPL activity under different conditions and in a variety of biological samples. The NBD fluorescence assay provides a reliable alternative to the standard radioactive assay. 

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