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Sphingosine-1-phosphate lyase has a central role in the development of *Dictyostelium discoideum*

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SUMMARY

Sphingosine-1-phosphate, a product of sphingomyelin degradation, is an important element of signal transduction pathways that regulate cell proliferation and cell death. We have demonstrated additional roles for sphingosine-1-phosphate in growth and multicellular development. The specific disruption in *Dictyostelium discoideum* of the sphingosine-1-phosphate lyase gene, which encodes the enzyme that catalyzes sphingosine-1-phosphate degradation, results in a mutant strain with aberrant morphogenesis, as well as an increase in viability during stationary phase. The absence of sphingosine-1-phosphate lyase affects multiple stages throughout development, including the cytoskeletal architecture of aggregating cells, the ability to form migrating slugs, and the control of cell type-specific gene expression and terminal spore differentiation. This pleiotropic effect, which is due to the loss of sphingosine-1-phosphate lyase, establishes sphingolipids as pivotal regulatory molecules in a wide range of processes in multicellular development.

Key words: Cisplatin resistance, Sphingolipids, Cell motility, Morphogenesis, Gene expression, Actin, Pseudopodia, EDG receptors, G proteins, GPI anchor, *Dictyostelium discoideum*

INTRODUCTION

Sphingolipids are emerging as important components in signal transduction. Ceramide and sphingosine-1-phosphate (S-1-P) are both components of the pathway of sphingomyelin degradation (Fig. 1). Both of these molecules have profound regulatory roles in controlling cell proliferation and cell death (Spiegel, 1999). It is believed that the relative levels of S-1-P and ceramide in the cell act as a rheostat that controls whether a cell divides or enters a cell death pathway. Increased levels of ceramide lead to cell death, while increased levels of S-1-P promote cell proliferation and inhibition of apoptosis (Igarashi, 1997; Spiegel, 1999). In addition, S-1-P has been shown to play roles in the release of intracellular Ca^{2+} stores, actin polymerization, cell motility, chemotaxis and cell differentiation, although the full extent of involvement of these molecules in multicellular development remains unknown (Pyne and Pyne, 2000).

In animal cells, there is strong evidence supporting an extracellular signaling role for S-1-P through a family of G-protein-coupled, seven transmembrane domain EDG receptors (endothelial differentiation growth; Pyne and Pyne, 2000; Spiegel and Milstien, 2000a; Spiegel and Milstien, 2000b; Van Brocklyn et al., 1998). Exogenously added S-1-P has been shown to inhibit cell motility and chemotactic invasiveness of various cell types in a pertussis toxin-sensitive manner (Sadahira et al., 1992; Sliva et al., 2000; Wang et al., 1999a; Yamamura et al., 1996). Ca^{2+} mobilization by S-1-P has been correlated with the expression of the EDG1 receptor in HEK 293 cells (Van Brocklyn et al., 1998) and in Chinese hamster ovary cells (Okamoto et al., 1998). Likewise, pertussis toxin-sensitive EDG1 expression is required for extracellular regulated kinase (ERK) activation by S-1-P in cultured airway smooth muscle cells (Pyne and Pyne, 1996; Wu, 1995).

There is growing support for an intracellular second messenger role for S-1-P as well. Microinjection of Swiss 3T3 cells with S-1-P induces DNA synthesis (Van Brocklyn et al., 1998), and the sphingosine kinase inhibitor D,L-threo-dihydrosphingosine blocks platelet derived growth factor stimulated S-1-P accumulation and ERK activation (Rani et al., 1997; Tolan et al., 1997). More importantly, overexpression of the S-1-P kinase in NIH 3T3 cells results in an increase in S-1-P, and a concomitant increase in DNA synthesis, cell growth and cell number. In addition, overexpression of S-1-P kinase protects the cells from ceramide-induced cell death. Importantly, no S-1-P was detected in the medium, indicating that S-1-P was functioning intracellularly (Kohama et al., 1998). Although the precise underlying mechanisms remain elusive, it is evident that the enzymes that modulate the concentration of these bioactive sphingolipids are important regulatory elements, controlling cell fate decisions and development.

Dictyostelium discoideum is an attractive organism to use for the study of cell and developmental biology, and the complex program of gene expression that underlies and controls cell-

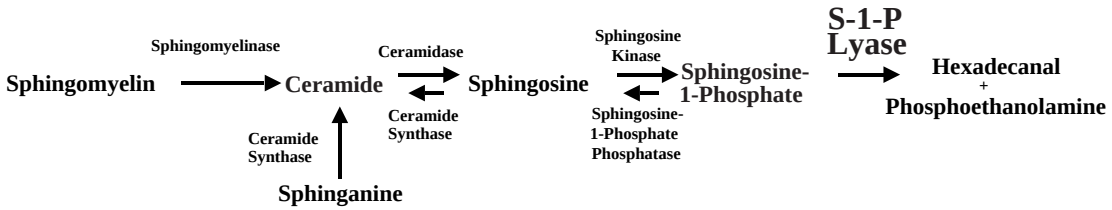

Fig. 1. Pathway of sphingomyelin degradation. The pathway reflects studies in animal cells. To date the genes for the S-1-P lyase (Accession Number, AF233610), two sphingosine kinases (cDNA clone SLG787 – Accession Numbers, AU061963 and AU039939, and cDNA clone SLE414 – Accession Number, AU061484), two sphingosine-1-P phosphatases (cDNA clone SSE389 – Accession Numbers, AU072386 and AU037870, and cDNA clone SSC687 – Accession Number, C85040) and a ceramidase (Accession Number, AAB69633) have been identified in *Dictyostelium*.

type differentiation and morphogenesis in this organism is becoming increasingly well understood (Kessin, 2001). Cells divide mitotically and remain as single cells as long as there is adequate food. When the food is depleted, the cells initiate the developmental program and groups of 1×10^5 cells aggregate chemotactically to form multicellular structures. These aggregates then proceed synchronously through the morphogenetic program, culminating in the construction of fruiting bodies, each with a mass of 80,000 spores, resting atop a slender multicellular stalk. The roles of cAMP, G-protein-coupled receptors and phosphoinositides in controlling cell motility, chemotaxis and gene expression have been intensively investigated (Laurence and Firtel, 1999; Parent and Devreotes, 1999).

We recently used the REMI (restriction enzyme mediated integration) insertional mutagenesis technique to identify *Dictyostelium* mutants with increased resistance to the widely used anticancer drug cisplatin (Li et al., 2000). One of the resistant mutants identified in the study possessed a disrupted *sgl*A gene, which encodes S-1-P lyase. The S-1-P lyase enzyme acts at the last step in sphingomyelin catabolism, and is responsible for the degradation of S-1-P to phosphoethanolamine and hexadecanal (Fig. 1; van Veldhoven and Mannaerts, 1993). This was the first identification of a component of this pathway in *Dictyostelium*, as well as the first implication of the involvement of this enzyme in cisplatin resistance.

In addition to resistance to cisplatin, the S-1-P lyase null mutant had a strong developmental phenotype and produced aberrant fruiting bodies, with short thick stalks and no obvious apical spore mass (Li et al., 2000). Previously, the S-1-P lyase gene had only been deleted in the yeast *Saccharomyces cerevisiae* (Gottlieb et al., 1999). Using the *Dictyostelium* S-1-P lyase null mutant, we have been able to examine the role of this enzyme in multicellular development. Our results show that S-1-P lyase functions at multiple steps throughout the course of development, including the cytoskeletal architecture of aggregating cells, slug migration, developmental gene expression and spore differentiation. In addition, cells that lack S-1-P lyase show increased viability in stationary phase. This pleiotropic effect, which is due to the loss of S-1-P lyase, establishes sphingolipids as central regulatory molecules in *Dictyostelium* growth and development.

MATERIALS AND METHODS

Assembly of the *sgl*A contig

The sequence of the *Dictyostelium sgl*A gene was assembled from information obtained from our sequencing (GenBank Accession Number, AF233610), from the cDNA sequencing project, Tsukuba, Japan (Morio et al., 1998) clone SLF575 (GenBank Accession Numbers, AU061728 and AU053046) and from contig #JC2a168e11.r1 from the genomic DNA sequencing project (http://genome.imb-jena.de/dictyostelium/). A 189 bp intron was found at position 112 from the ATG.

Homologous disruption of the *sgl*A gene

An 831 bp fragment of *sgl*A was amplified from genomic DNA by PCR, using the 5′ primer, 5′-CTAGTCTAGAGTTTCCC-ATCAATTCG (#225) and the 3′ primer, 5′-CAAGAGAATGC-AACAACC (#226). The amplified DNA fragment was digested with *Xba*I and *Xho*I and cloned into *Xba*I-*Xho*I digested pBluescript KS(+) to generate plasmid pCis2B. A 1.4 kb *Bam*HI cassette, containing the blasticidin S resistance (bsr) gene (Kamakura et al., 1987), was purified from plasmid SL63 (obtained from R. Firtel, University of California, San Diego, CA) and inserted into the *Bam*HI site within the *sgl*A-coding region, to generate plasmid Rcis2B, to be used for gene disruption. An *Xba*I-*Xho*I fragment from Rcis2B containing the bsr gene was used for disruption of the endogenous *sgl*A gene.

DNA transformation and selection were performed as described (Kuspa and Loomis, 1992). Briefly, 1.2×10^7 Ax4 cells were transformed with 20 μg of excised DNA fragment at 1kV, 3 μF in 4 mm-gap-width cuvettes using the BioRad Gene pulser. Cells were cured with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ for 10 minutes, and then were resuspended in 40 ml HL5 medium and transferred to petri dishes. After 24 hours, 10 μg/ml blasticidin S (ICN, Costa Mesa, CA) was added and the plates were incubated at 22°C. Colonies began to appear after 7-10 days. To isolate single clones, transformed cells were plated on SM agar plates with *Klebsiella aerogenes* (Sussman, 1987).

All standard DNA manipulations were performed according to published methods (Sambrook et al., 1989). Genomic DNA was prepared using DNAzol reagent, and total RNA was isolated using TRI reagent (both from Molecular Research Center, Cincinnati, OH). PCR fragments were purified using GENECLEAN III (BIO 101, Vista, CA).

Southern and northern analyses

Southern and Northern analyses were performed as described (Lee et al., 1997). DNA (20 μg/lane) or total RNA (10 μg/lane) were used for Southern and northern analyses, respectively. Equal loading of RNA was monitored by staining for rRNA with 0.25 μg/ml Acridine Orange. Northern blots were quantified by using a FUJIFILM FLA-2000 PhosphorImager. Southern nylon blots were stripped by pouring a boiling solution of 0.1×SSC (15 mM NaCl, 1.5 mM $Na_3C_6H_5O_7\cdot2H_2O$, pH 7.0) containing 0.1% SDS over the blot in a Pyrex dish. The blots were shaken slowly for 15 minutes at room temperature, and the procedure was repeated three times. To test for complete removal of the labeled probe, the blots were re-scanned using the PhosphorImager. The following hybridization probes were used: *sgl*A, a 0.83 kb PCR product from genomic DNA; *bsr*, a 1.4 kb

*Bam*HI fragment from SL63; CSA, c512 plasmid, linearized with *Bgl*II; *ecm*B, a 2.4 kb *Hin*dIII fragment from pDd56; PsA, a D19 plasmid, linearized with *Nsi*I; *cat*Bl, a 2.1 kb *Sal*I-*Not*I fragment from SLK452; and *spi*A, a 0.45 kb PCR product amplified from genomic DNA.

Strains and conditions for growth and development

Ax4 was the parental strain used for the generation of the *sgl*A mutant by direct homologous recombination. Cells were grown in HL-5 medium and passed, or used for experiments, when the cell density reached 2-3×10^6 cells/ml (Cocucci and Sussman, 1970). New cultures were started monthly from stored spores. Cell numbers was monitored by counting in a hemacytometer. For development, cells were harvested and washed in LPS buffer (20 mM KCl, 2.5 mM $MgCl_2$, 40 mM K-PO_4, pH 6.5 containing 0.5 mg/ml streptomycin sulfate). Aliquots of 1×10^8 washed cells were deposited on 40 mm diameter black paper filters (Thomas Scientific) supported by LPS buffer-saturated Gelman cellulose filter pads, and were allowed to develop at 22°C (Soll, 1987; Sussman, 1987). The cells on each filter aggregate to form approximately 1000 multicellular assemblies, which proceeded through the remainder of development in synchrony. Photographs were taken at hourly intervals to record morphogenetic changes. Developing cells were harvested by vortexing the filters in 2 ml SS buffer (10 mM NaCl, 10 mM KCl, 2.7 mM $CaCl_2$). The samples were either used directly for experiments or frozen as pellets for later molecular analyses.

Immunofluorescence staining

Cells were allowed to develop on black paper filters until the onset of aggregation at 5 hours. The aggregating cells were harvested, disaggregated by vortexing and washed twice in LPS buffer. The washed cells were deposited at 2×10^4 cells/cm^2 on ethanol-washed coverslips. After 10 minutes, the coverslips were flooded with a fixative solution of 3.7% formaldehyde in LPS, and incubated for 5 minutes at room temperature. To permeabilize the cells, the fixative solution was replaced with a solution of 0.5% NP-40 in LPS, and incubated for 5 additional minutes. The coverslips were then washed three times with LPS, and incubated overnight in 1% bovine serum albumin (BSA) in LPS at 4°C (Alexander et al., 1992). For F-actin staining, cells were incubated for 20 minutes with 25 nM rhodamine-labeled phalloidin in 1% BSA/LPS. Coverslips were then washed three times with LPS and mounted in a solution of Airvol (Air products, Allentown, Pa., USA) containing 50 mg/ml DABCO (1,4-diazabicyclo[2-2-2]octane) to prevent fading of fluorescence. Slides from four different experiments were examined and photographed with a Zeiss IM inverted microscope using a 100× Neofluor lens.

Slug migration and phototaxis assay

Cells were grown axenically to a density of 2-3×10^6 cells/ml, harvested by centrifugation, washed twice with water and suspended in water to a final concentration of 5×10^8 cell/ml. 0.1 ml of this suspension was deposited as a 3 cm line at one end of a 1.5% H_2O-agar plate. The plates were wrapped in foil, a pin hole was made in the foil at the end opposite to where the cells were deposited and the plates were incubated at 22°C in a lighted incubator (Newell et al., 1969). After two days, the plates were removed and the slugs and slime trails were lifted onto 0.2 mm thick polyvinylchloride discs, which were then air dried, stained for 30 seconds with 0.3% (w/v) Coomassie Blue in 50% ethanol/10% acetic acid, washed and blotted dry for a permanent record of the experiment (Fisher et al., 1981).

Sporulation efficiency assay

Mutants and wild-type cells were allowed to develop on black paper filters. At 24 and 48 hours, the cells were collected by vortexing the filters in 2 ml SS buffer. Spores and total cells from each sample were counted using a hemacytometer. The sporulation efficiency is defined as the percentage of the spores in the total cells counted.

S-1-P treatment

A stock solution of S-1-P was prepared by dissolving the S-1-P to 0.5 mg/ml in methanol according to the supplier's instructions (Sigma, St Louis, MO). The methanol was evaporated by streaming nitrogen through the tube, and the powder was dissolved in 4 mg/ml fatty acid-free BSA in water to a final concentration of 125 μM. Wild-type Ax4 cells were allowed to develop on LPS saturated filters. At 5, 8 and 12 hours, filters were transferred to new pads saturated with either 5, 10 or 50 μM S-1-P in LPS, and development was allowed to proceed. The filters were photographed at 24 and 60 hours of development to characterize morphogenesis.

RESULTS

The S-1-P lyase gene

The *Dictyostelium* S-1-P lyase gene was identified initially during a selection for cisplatin resistant mutants following REMI insertional mutagenesis (Li et al., 2000). We have now assembled the sequence of the entire *sgl*A gene from sequences in the cDNA and the genomic sequencing project databases and our own sequencing (see Materials and Methods). Comparison of the predicted *Dictyostelium sgl*A gene product with sequences of other S-1-P lyase proteins reveals that the S-1-P lyase protein is highly conserved throughout the majority of the molecule, and shows 40% identity (61% similarity) to the yeast, and 42% identity (62% similarity) to the mouse and human genes (Fig. 2).

Northern analysis showed that the level of *sgl*A mRNA is maintained at a constant steady state level throughout both growth and development (Fig. 3). The presence of *sgl*A mRNA throughout the life cycle is consistent with the pleiotropic effects observed with the *sgl*A-null mutant during both growth and development.

Generation of S-1-P lyase null mutant

The original REMI insertional mutant was recapitulated by direct homologous recombination as described in Materials and Methods and Fig. 4A. Southern analysis confirmed that this was due to a single homologous recombination event, which resulted in a single insertion of the blasticidin-resistance cassette into the *Bam*HI site (position 687) within the 0.1 kb *Cla*I fragments of the *sgl*A gene (Fig. 4B). Northern analysis showed a 1.9 kb transcript in the wild-type, and a truncated mRNA, which terminates within the insertion sequence, in the *sgl*A-null mutant, (Fig. 4C). The truncated message could encode less than 40% of the gene, in a region of low homology (see Fig. 2), and was expressed at a considerably lower level (less than 30% of the level in wild type). The mutant that resulted from the direct disruption exhibited the same aberrant developmental phenotypes, as well as the same level of resistance to cisplatin, that we observed with the original REMI mutant (Li et al., 2000).

Morphogenesis in the S-1-P lyase null mutant is abnormal

We previously demonstrated that the exponential growth rate in axenic culture of the S-1-P lyase null mutant was the same as the parental wild-type cells (Li et al., 2000). In Fig. 5A we show now that the null mutant has greater viability in stationary phase than the wild type. Both mutant and wild type grow to equivalent cell densities. The wild-type cells rapidly die (the

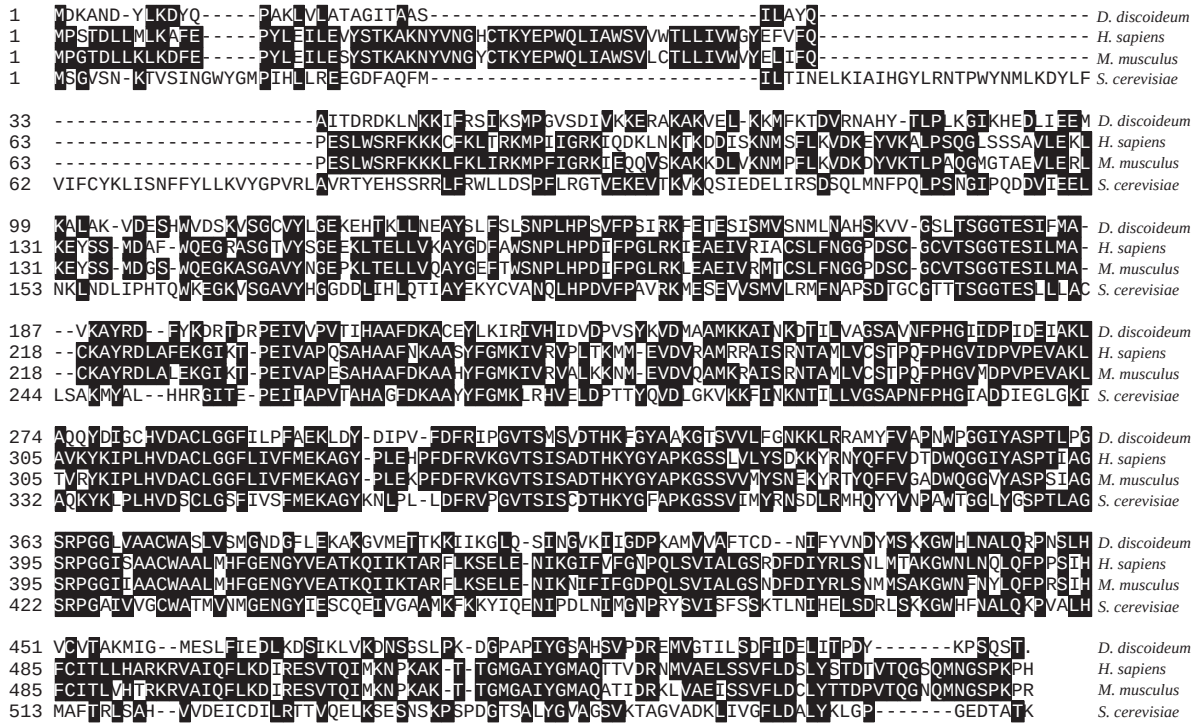

Fig. 2. Sequence homology of S-1-P lyase gene product. The sequence information of the S-1-P lyase contig was assembled by combining sequence information from the various *Dictyostelium* sequencing project databases (see Materials and Methods). The predicted amino acid sequence of *Dictyostelium* S-1-P lyase was aligned with the human, mouse and *Saccharomyces cerevisiae* S-1-P lyase gene products (NCBI Accession Numbers, AAD44755, NP033189 and NP010580, respectively). Alignment was performed using the MegAlign program of DNASTAR.

cell number decreases, owing to cell lysis), in contrast to the mutant cells, which die at a much slower rate, lagging the wild type by 3 days. We also examined whether mutant cells were capable of cell division during development; Fig. 5B shows that both they and wild-type cells show an equally modest increase in cell number during the first 12 hours of development. This increase was less than that reported previously for Ax2 cells (Zada-Hames and Ashworth, 1978).

Our original observations of this mutant showed that it formed aberrant terminal developmental structures with short thick stalk and a bulbous end. We have now carefully examined the entire timecourse of development under conditions where morphogenesis is synchronous. Fig. 6 depicts the developmental time course of the parent Ax4 and the S-1-P lyase null strains photographed at intervals throughout development. Both mutant and wild-type strains reach the ripple stage by 5 hours, and form tight aggregates by 8 hours. In multiple experiments, the mutant proceeds through early development slightly faster than its parent. The mutant aggregates are larger than the wild type, and they eventually break apart into smaller aggregates (seen at 12 hours). The mutant aggregates are unable to form the elongated slug morphology (typical of 16 hours) and proceed slowly over the next 20 hours to ultimately form the aberrant terminal structures. By contrast, the wild-type parent develops through the slug stage, and proceeds through the rest of development at a normal rate, producing normal fruiting bodies at 24 hours. In repeated experiments, development of the mutant was highly synchronous until after aggregation, when they become

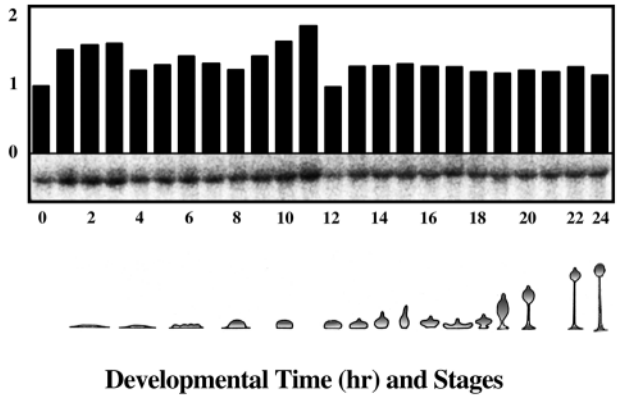

Fig. 3. Expression of *sgl*A gene during growth and development. Ax4 wild-type cells were allowed to develop synchronously on filters. Total RNA samples were prepared at the indicated time points and analyzed for *sgl*A mRNA levels. The band intensities on the northern blots were quantified with a FUJIFILM FLA-2000 PhosphorImager. The mRNA levels are depicted as -fold increase over the mRNA level at 0 hours of development.

blocked. Further incubation of the filters up to 60 hours did not result in additional morphogenesis. It should be noted that within the population of mutant multicellular aggregates there are a small number of ‘normal’ looking structures. This most likely reflects a lack of complete penetrance of this mutation (see Discussion).

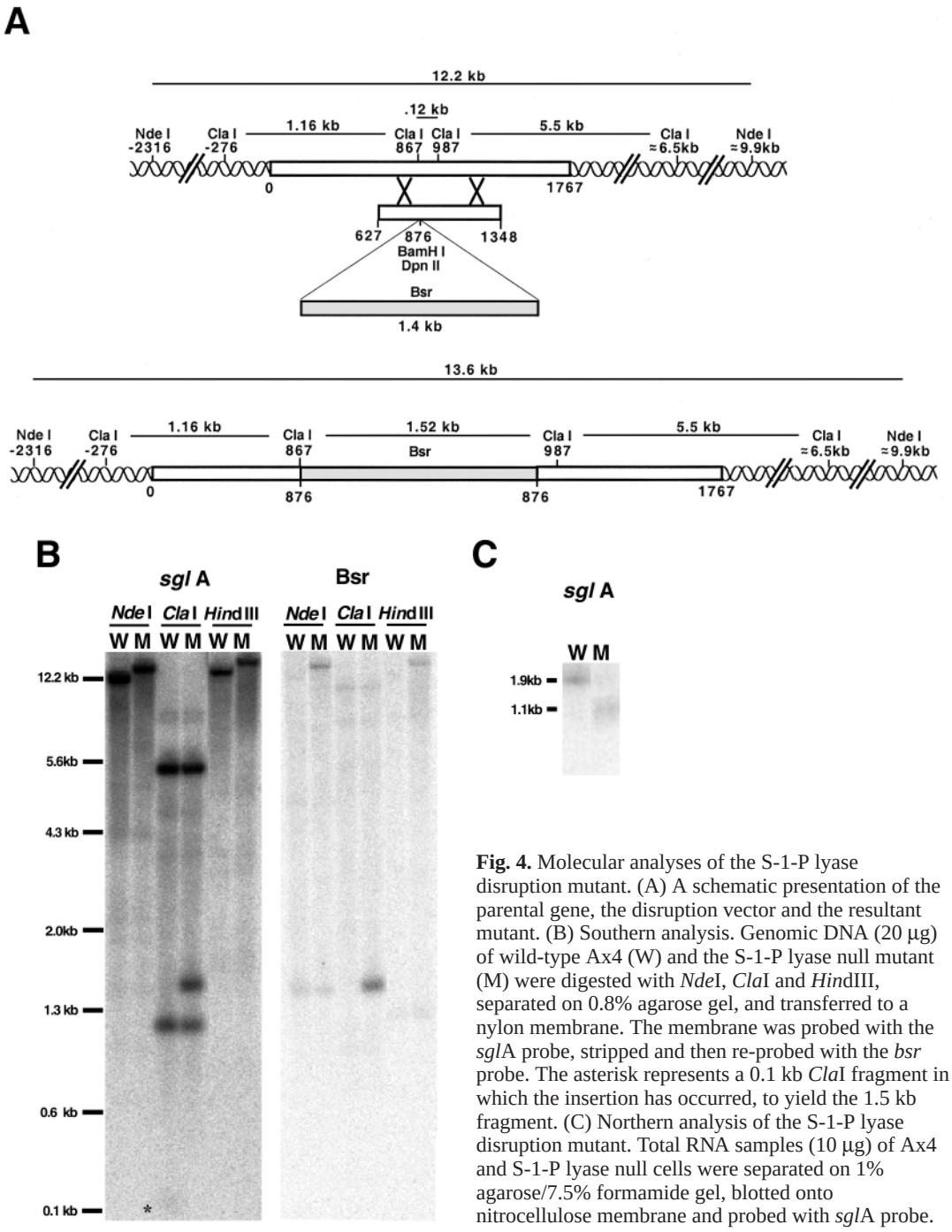

Fig. 4. Molecular analyses of the S-1-P lyase disruption mutant. (A) A schematic presentation of the parental gene, the disruption vector and the resultant mutant. (B) Southern analysis. Genomic DNA (20 μg) of wild-type Ax4 (W) and the S-1-P lyase null mutant (M) were digested with *Nde*I, *Cla*I and *Hin*dIII, separated on 0.8% agarose gel, and transferred to a nylon membrane. The membrane was probed with the *sgl*A probe, stripped and then re-probed with the *bsr* probe. The asterisk represents a 0.1 kb *Cla*I fragment in which the insertion has occurred, to yield the 1.5 kb fragment. (C) Northern analysis of the S-1-P lyase disruption mutant. Total RNA samples (10 μg) of Ax4 and S-1-P lyase null cells were separated on 1% agarose/7.5% formamide gel, blotted onto nitrocellulose membrane and probed with *sgl*A probe.

Spore differentiation in the S-1-P lyase null strain is reduced

The S-1-P lyase null mutants did not form an obvious spore mass at the end of morphogenesis. To determine if spores were being produced, we harvested aggregates from mutant and wild-type strains at 24 and 48 hours of development, disaggregated them and examined the cell populations for the presence of mature, elliptical, phase-bright spores. The mutant possessed no visible spores at 24 hours, and only about 30% spores at 48 hours. This is in contrast to the wild type, which has roughly 65% spores at 24 hours and 70% at 48 hours. The data presented in Fig. 7 demonstrate that spore production in the S-1-P lyase null strain is reduced compared with the wild-type parent. The spores that are made are viable, and maintain their viability and ability to germinate after storage for up to a year by freezing or desiccation. We, and others, have shown that spores with compromised viability do not store for long periods of time (Richardson and Loomis, 1992; Srinivasan et al., 2000).

Slug migration is defective in the S-1-P lyase null strain

Our examinations of the timecourse of development (Fig. 6)

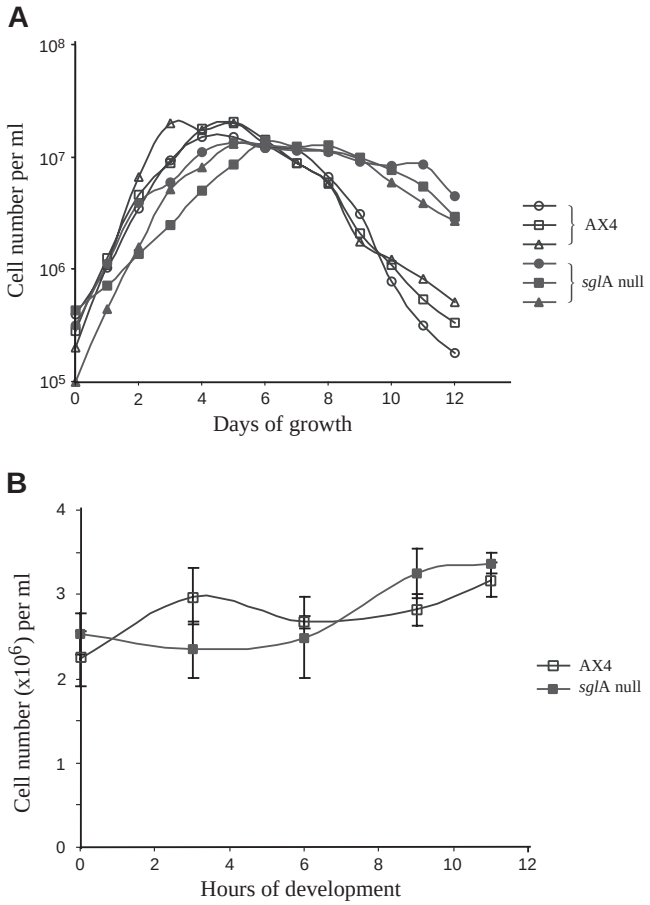

Fig. 5. Disruption of the S-1-P lyase gene affects viability of cells in stationary phase. (A) Wild-type and S-1-P lyase-null cells were inoculated into HL-5 medium at 2×10^5 cells per ml for axenic growth and the cell numbers were followed. Triplicate cultures of each were examined. Each point is the mean of duplicate counts. (B) Wild-type and S-1-P lyase cells were harvested at a density of 3×10^6 cells per ml and deposited at a standard density of 8×10^6 cells/cm^2 for development. At the indicated times, duplicate filters were harvested into 20 ml of SS buffer containing 10 mM EDTA and disaggregated by vortexing until there was a uniform suspension of single cells. The cells from each filter were counted in duplicate and the bars in the figure indicate the standard deviation.

Aggregating S-1-P lyase null cells show aberrant actin distribution and pseudopodia formation

The cytoskeletal architecture and motile behavior of *Dictyostelium* cells have been studied extensively (Wessels and Soll, 1998). Because S-1-P lyase is involved in metabolism of the membrane component sphingomyelin, we wanted to determine if the absence of S-1-P lyase had a direct effect on cell morphology and cytoskeletal architecture during the aggregation stage. We stained aggregation-competent wild-type and mutant cells with rhodamine-labeled phalloidin and examined the shape and F-actin distribution of the cells. Fig. 9A shows wild-type cells, which are characteristically elongated with large anterior F-actin filled pseudopods, tapered posterior uropods and frequent filopodia extending around their periphery. By contrast, the S-1-P lysase null mutant cells (Fig. 9B) are less elongated and appear smaller. They often appear less flat and with less elongated uropods than the wild type. Filopodia are almost entirely absent from the mutant cells. Most striking is the atypical organization of F-actin, where there is a distinct lack

suggested that the mutant is unable to form migrating slugs. We directly tested this by allowing cells to develop under conditions that favor slug formation and migration (Newell et al., 1969). The results in Fig. 8A show that the parental wild-type strain readily forms slugs that are capable of directed migration towards a light source. By contrast, aggregates of the S-1-P lyase null strain developed directly at the site of aggregation (Fig. 8B). A small number of aggregates are able to form slugs that show extremely limited directional migration before stopping. Again, this is probably due to incomplete penetrance. The mutant is not phototactically compromised (blind), as 'blind' mutants are able to migrate over a long distance, albeit without direction (Darcy et al., 1994).

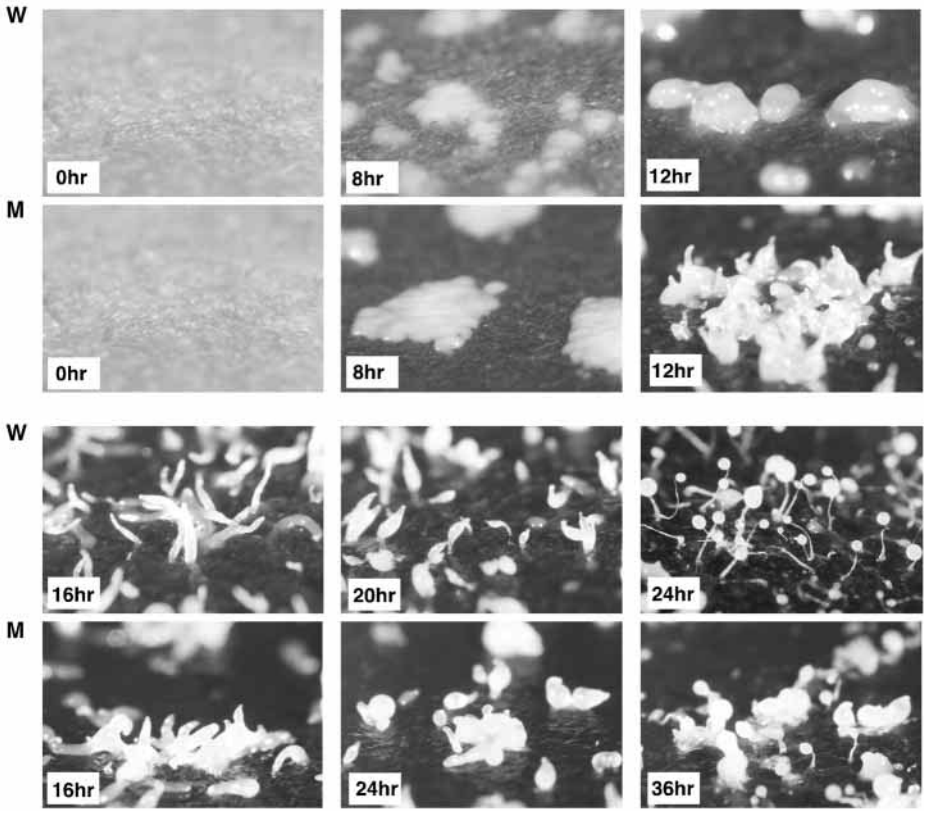

Fig. 6. The S-1-P lyase null strain has abnormal development. Axenically grown wild-type Ax4 and S-1-P lyase null cells were allowed to develop on filters and were photographed at the indicated times. 1 cm=4.2 mm.

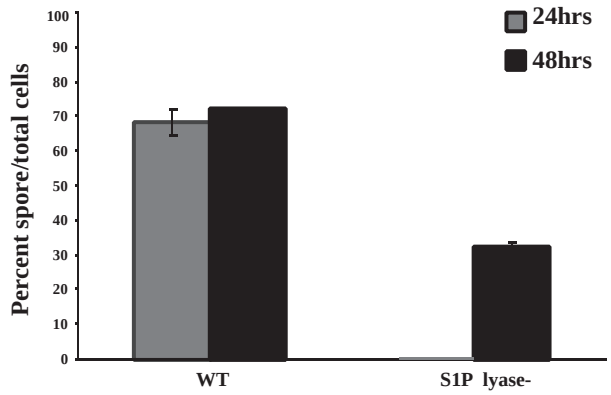

Fig. 7. Sporulation is reduced in the S-1-P lyase-null strain. Wild-type Ax4 and S-1-P lyase-null cells were allowed to develop synchronously on filters. Samples were harvested at indicated time points and analyzed for the percentage of spores out of total number of cells counted.

of large F-actin filled pseudopods in the anterior end of the mutant cells. In many cells, the F-actin appears localized to the interior of the cells (Fig. 9B, panels 1, 4, 6, 8, 9), although there are cells that have greatly reduced levels at their anterior ends (Fig. 9B, panel 2) or localized around the entire cell (Fig. 9B, panels 4, 7).

The expression of developmental specific genes reflects the abnormal morphogenetic phenotype

The delayed developmental schedule, coupled with the impaired ability of the mutant strain to form migrating slugs or produce spores, suggested a corresponding change in the pattern of expression of developmentally regulated genes. Fig. 10 shows the expression profile of several genes that are landmarks of early, middle and late development. Contact site A (CSA), which is the cell adhesion molecule gp-80, is expressed at the onset of development (Muller and Gerisch, 1978). This gene was expressed in the mutant in relatively normal fashion, though it appeared to occur slightly earlier and extended a little longer, consistent with the slightly earlier aggregation observed in the mutant, but overall the pattern is very similar to that seen with the parent strain.

*ecm*B and D19 are both genes that are normally expressed later in development, at the beginning of the slug stage, and are cell-type specific. The D19 gene is expressed only in prespore cells and encodes the membrane-bound PsA protein (Early et al., 1988), while *ecm*B is a stalk-specific marker that encodes the large glycoprotein ST310, which becomes part of the stalk (McRobbie et al., 1988). The results show that the inability of the mutant to make slugs does not affect the onset of expression of these genes. However, the duration of their expression is extended in the mutant, presumably reflecting the stall in development in this strain.

Last, we tested two genes that are expressed at the terminal stage of development of prespore cells. The expression of the *cat*B gene, which encodes the catalase B enzyme, which is expressed only in prespore cells (Garcia et al., 2000), and the *spi*A gene, which encodes a spore coat protein (Richardson and Loomis, 1992), is drastically reduced in the mutant. The diminished expression of these genes is consistent with the diminished ability of the mutant to complete morphogenesis and spore differentiation, though we cannot distinguish between diminished expression in all the cells versus normal expression in a small fraction of the cells.

Addition of extracellular S-1-P generates a phenocopy of the *sgl*A null strain

The lack of S-1-P lyase suggested that the proximal cause of the developmental defects in the mutant were due to an increase in S-1-P. To examine this, we treated developing wild-type cells with extracellular S-1-P, added at different times during development (5, 8 and 12 hours), and followed the fate of the developing aggregates. Adding 5 or 10 μM S-1-P had no effect (data not shown). The addition of 50 μM S-1-P to wild-type cells led to abnormal development, which mimicked that of the S-1-P lyase null mutant (Fig. 11). The effect became more pronounced when the S-1-P was added at the later time points, and was not altered by prolonged incubation of up to 60 hours.

These results demonstrate that extracellular S-1-P can affect development, and suggest that relatively high concentrations of S-1-P are needed to reproduce this phenotype. This notion is supported by experiments in which mixtures of mutant and wild-type cells were allowed to develop together. A mixture of 90% mutant and 10% wild-type cells developed with a mutant phenotype, as expected. A mixture of 10% mutant and 90% wild-type developed as wild type. This suggests that S-1-P is not secreted from the S-1-P lyase-null cells in any appreciable concentrations during the course of development, and that 10% of mutant population could not overcome the wild-type phenotype.

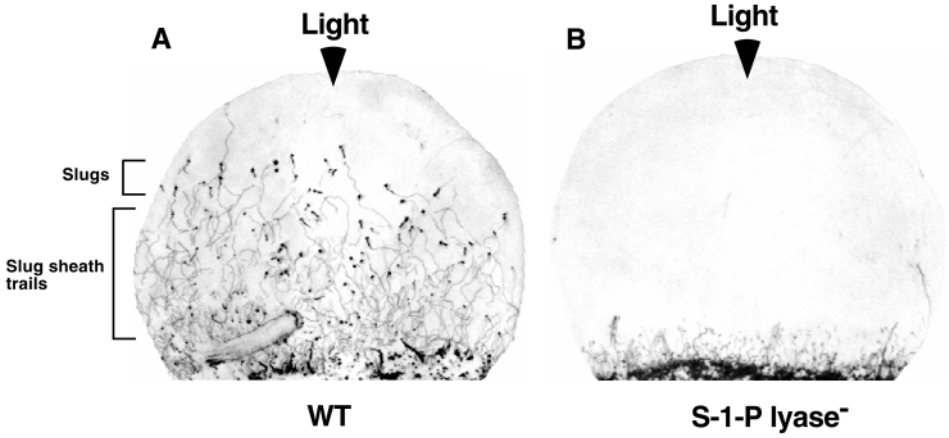

Fig. 8. Migration is impaired in S-1-P lyase null slugs. The wild-type Ax4 (A) and S-1-P lyase null (B) cells were grown axenically and harvested for the slug migration assay as described in the Materials and Methods. Gray lines represent the trails of slime sheath, which are left behind during the migration of the slug during the 2 day period; dark dots indicate the final position of the migrating slug. The position of the light source is indicated by the arrowhead.

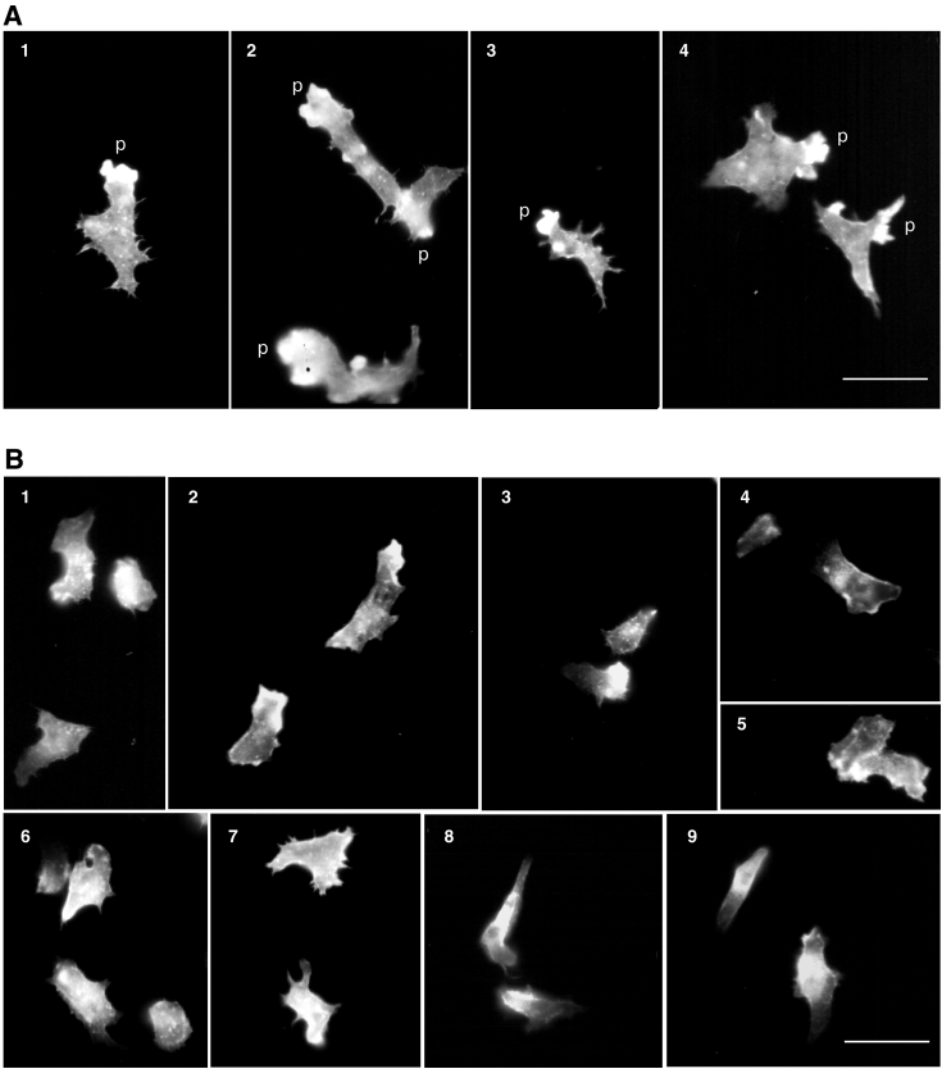

Fig. 9. Aggregating S-1-P lyase mutant cells have altered shape and aberrant cell F-actin localization. Aggregating wild-type Ax4 (A) and S-1-P lyase null (B) cells were prepared as described in the Materials and Methods, and stained with rhodamine-conjugated phalloidin. Scale bars: 10 mm.

DISCUSSION

In a previous study, we used insertional mutagenesis in *Dictyostelium discoideum* to identify mutants that had increased resistance to the widely used anticancer drug cisplatin (Li et al., 2000). One of the novel resistant mutants that we recovered in this selection was the result of the disruption of the *Dictyostelium sgl*A gene, encoding the S-1-P lyase. In animal cells, the S-1-P lyase is known to be a downstream component in the pathway of sphingomyelin degradation and converts S-1-P to phosphoethanolamine and hexadecanal. Neither the S-1-P lyase nor other genes in this pathway has been previously studied in *Dictyostelium*.

The disruption of the S-1-P lyase probably results in elevated levels of S-1-P in the *Dictyostelium* cells. This is supported by the observation that platelets, which naturally lack S-1-P lyase activity, accumulate high levels of S-1-P (Yatomi et al., 1997), and by the observation that disruption of the yeast S-1-P lyase, *BST1*/*DPL1*, resulted in increased levels of S-1-P (Gottlieb et al., 1999; Saba et al., 1997). Thus, we have suggested that the disruption of the S-1-P lyase in our mutant results in an increase in S-1-P levels that counteracts the cytotoxic effects of cisplatin by promoting cell proliferation and inhibiting the induction of cell death (Li et al., 2000). Accordingly, we have now shown that deletion of the S-1-P lyase gene in *Dictyostelium* results in increased viability in stationary phase. In yeast, it has been shown that deletion of *BST1*/*DPL1* results in the mutant cells growing to a higher cell density before entering stationary phase (Gottlieb et al., 1999). Thus, in both organisms, the deletion of this gene appears to confer a growth phase advantage.

Much less is known about the role of S-1-P in cell differentiation and multicellular development. Thus, it was significant that disruption of the *Dictyostelium* S-1-P lyase resulted in aberrant development. We have now analyzed this mutation throughout the entire developmental program. The data support the idea that its effects are highly pleiotropic,

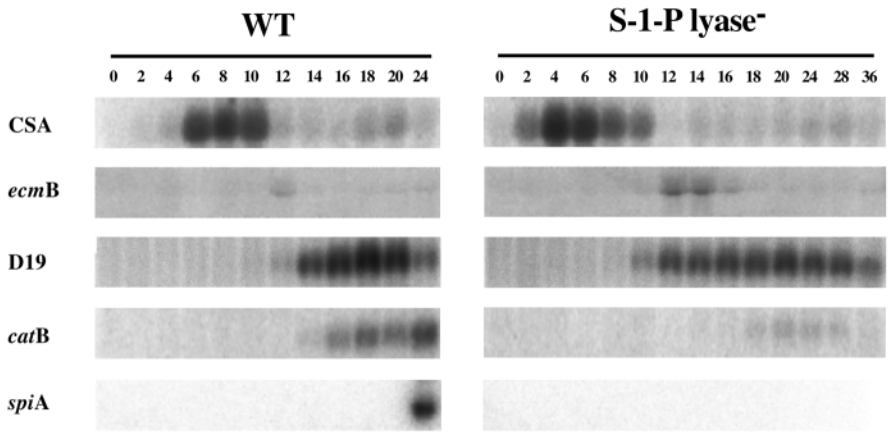

Fig. 10. Late developmental gene expression is altered in the S-1-P lyase-null strain. Wild-type Ax4 and S-1-P lyase null cells developing synchronously on filters were harvested. Northern blots of total RNA prepared from each time point were probed for the expression of different developmental marker genes using gene-specific probes.

showing involvement throughout development, including the distribution of F-actin in aggregating cells, the ability to form migrating slugs, late developmental gene expression, and the ability to complete morphogenesis and spore differentiation. This array of phenotypes is consistent with the S-1-P lyase gene product functioning in a wide range of processes within the cell, and in accord with the expression of S-1-P lyase mRNA at a constant level throughout both mitotic growth and development.

The data indicate that the S-1-P lyase-null mutant is not entirely penetrant: some individual structures within the population do not display the mutant phenotype. Thus, a small percentage of aggregating cells have F-actin-filled pseudopods, some multicellular aggregates can make slugs with limited motility, and some spore specific gene expression and spore differentiation occurs. This behavior is consistent with the idea that the phenotype is the result of an increase in S-1-P, rather than the lack of a metabolite, as is often the case with gene disruptions. Heterogeneity in phenotypes that is due to incomplete genetic penetrance has been observed in numerous systems, including in a variety of human diseases and cancers.

Because S-1-P lyase is involved in metabolism of membrane components, it must be considered that these phenotypes could be the result of a global nonspecific alteration to the membrane structure. It is possible that the effects on F-actin distribution may be mediated at least partially through the membrane (see below). However, we feel that changes in membrane structure are not likely to be the cause for the other phenotypes in the mutant, owing to the known signal transduction roles of S-1-P and to the specificity and the independence of the phenotypes observed.

The finding that S-1-P lyase-null cells have altered shape and F-actin distribution is particularly exciting and relates directly to pharmacological studies with human cells. Exogenously added S-1-P has been shown to inhibit cell motility and chemotactic invasiveness of B16 melanoma (Sadahira et al., 1992) and estrogen-independent MDA-MB-231 invasive breast cancer cells (Sliva et al., 2000; Wang et al., 1999a). In addition, S-1-P inhibits motility of normal smooth muscle cells (Sadahira et al., 1992) and chemoattraction of neutrophils to interleukin 8 (Yamamura et al., 1996). Some data support the idea that S-1-P acts extracellularly through cell surface receptors to inhibit cell motility (Yamamura et al., 1997), although other studies support an intracellular mechanism for S-1-P. They show no involvement of EDG receptors, and that overexpression of the S-1-P kinase, which generates S-1-P, alters motility (Wang et al., 1999b). These changes in cell behavior are accompanied by a reduction in F-actin nucleation and pseudopod formation (Wang et al., 1999b; Yamamura et al., 1997). *Dictyostelium discoideum* has been the subject of intense investigation of cell motility and chemotaxis (Noegel et al., 1997; Parent and Devreotes, 1999), resulting in an unprecedented understanding of these responses in this organism. We are therefore well placed to use *Dictyostelium* to further elucidate the molecular roles of S-1-P in cell motility.

The inability to form migrating slugs is a dramatic phenotype that is associated with the disruption of the S-1-P lyase. It is possible that this defect is due to a fundamental defect in the motility of the individual cells, as reflected by the aberrant F-actin distribution. However, it is also possible that the end products of S-1-P degradation are needed for the synthesis of lipid components that are required for slugs to migrate. In this regard, it has been demonstrated that the *mod*B-dependent form of protein glycosylation is required for slug migration (Alexander et al., 1988). Interestingly, some of the *mod*B-modified proteins are also modified with a glycolipid anchor used for localization to the plasma membrane (Gooley et al., 1992). Indeed, phosphoethanolamine is a precursor

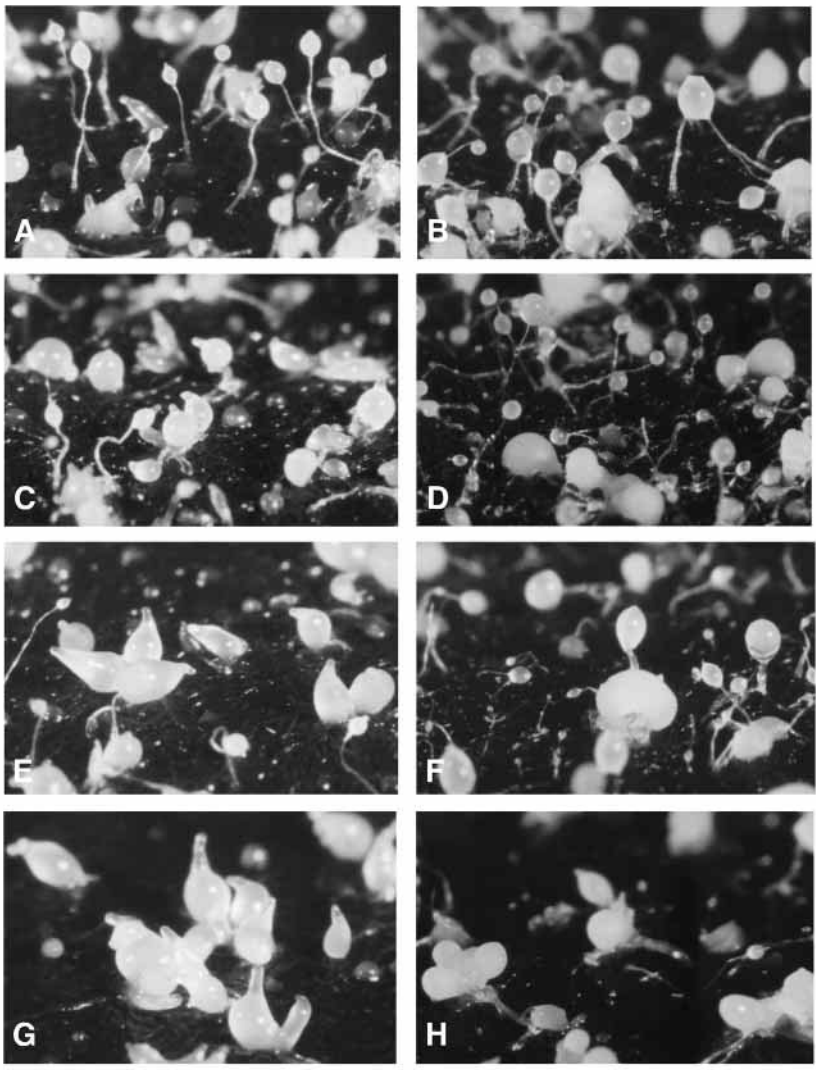

Fig. 11. S-1-P treatment of wild-type cells during development produces a phenocopy of the S-1-P lyase null mutant. Wild-type Ax4 cells were allowed to develop synchronously on filters (A,B). S-1-P was added at 5 (C,D), 8 (E,F) and 12 (G,H) hours of development, as described in Materials and Methods. The photographs were taken at 24 (A,C,E,G) and 60 (B,D,F,H) hours of development. 1 cm=2 mm.

of glycosylphosphatidylinositol, which functions as the membrane anchor (Canivenc-Gansel et al., 1998), and it is possible that the lipid anchors of these proteins are missing in the S-1-P lyase mutants, rendering the proteins nonfunctional.

The developmental timing of the S-1-P lyase null mutant is abnormal. The mutant aggregates slightly earlier than the wild type and is impaired in development. The expression of developmentally regulated genes reflects the phenotype, with a slightly premature expression of the CSA gene, and a lengthened period of expression of the *ecm*B and D19 genes. These results concur with many studies in *Dictyostelium* that have repeatedly demonstrated that the pattern of developmentally regulated gene expression is integrated with the overall progress of morphogenesis (Sussman and Brackenbury, 1976). That is, in slowly developing mutants, the periods of gene expression are lengthened, and in rapidly developing mutants, the periods are proportionally shortened.

The expression of both the catalase B gene and *spi*A spore coat protein gene is drastically reduced. Both these gene products are expressed at the very end of development in prespore cells. The absence of these gene products reflects that development in the mutant to the very late step of spore differentiation is impaired. The ability to form environmentally resistant spores is a major evolutionary advantage, and this result indicates the importance of the *sgl*A gene in the natural history of this organism.

Extensive studies in *Dictyostelium* have shown that cAMP activation of PKA is required for normal cell differentiation and morphogenesis (Loomis, 1998). In animal cells, it has been shown that S-1-P can modulate the levels of cAMP (through modulation of adenylate cyclase activity) and that cAMP can, in turn, modulate the levels of S-1-P (through cAMP-mediated activation of sphingosine kinase; Machwate et al., 1998; Van Brocklyn et al., 1998). This raises the possibility that some of the phenotypes that relate to spore specific gene expression and spore differentiation that we observe in the S-1-P lyase mutant may be mediated through the modulation of cAMP levels. Therefore, this work suggests another pathway for the regulation of developmental gene expression. However, changes in cAMP are probably not the underlying reason for other phenotypic changes in this mutant strain, including, for example, the F-actin distribution in single aggregation competent cells or the inhibition of slug migration.

Addition of exogenous S-1-P to wild-type cells mimics the overall aberrant developmental phenotype of the S-1-P lyase null mutant, and supports the idea that the mutant phenotypes result from increased levels of S-1-P in the cells. We do not know if the increased S-1-P level acts intracellularly or whether S-1-P is secreted and then acts in an autocrine manner. To date, the *Dictyostelium* genomic and cDNA sequencing projects have not identified an EDG receptor homolog in *Dictyostelium*. This may explain why relatively high levels of S-1-P are required to phenocopy the mutant, although *Dictyostelium* is well known for its relative resistance to a wide variety of drugs.

Overall, this study demonstrates that sphingolipids play a pivotal regulatory role in a wide range of processes in growth and multicellular development. This is reminiscent of the central regulatory role of cAMP during *Dictyostelium* development. Further work is required to elucidate all the details of how changes in sphingolipid metabolism affect development; the data presented here suggest that it has both structural and regulatory roles. To this end, we have recently identified other genes of this pathway, including two sphingosine kinases, two sphingosine-1-P phosphatases, an acid sphingomyelinase and a ceramidase, and we are currently constructing deletion and overexpression mutants with which to study the relationship between sphingolipids metabolism and multicellular development.

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REFERENCES

Alexander, S., Smith, E., Davis, L., Gooley, A., Por, S. B., Browne, L. and Williams, K. L. (1988). Characterization of an antigenically related family of cell-type specific proteins implicated in slug migration in *Dictyostelium discoideum*. *Differentiation* **38**, 82-90.

Alexander, S., Sydow, L. M., Wessels, D. and Soll, D. R. (1992). Discoidin proteins of *Dictyostelium* are necessary for normal cytoskeletal organization and cellular morphology during aggregation. *Differentiation* **51**, 149-161.

Canivenc-Gansel, E., Imhof, I., Reggiori, F., Burda, P., Conzelmann, A. and Benachour, A. (1998). GPI anchor biosynthesis in yeast: phosphoethanolamine is attached to the alpha1,4-linked mannose of the complete precursor glycophospholipid. *Glycobiology* **8**, 761-770.

Cocucci, S. and Sussman, M. (1970). RNA in cytoplasmic and nuclear fractions of cellular slime mold amebas. *J. Cell Biol.* **45**, 399-407.

Darcy, P. K., Wilczynska, Z. and Fisher, P. R. (1994). Genetic analysis of *Dictyostelium* slug phototaxis mutants. *Genetics* **137**, 977-985.

Early, A. E., Williams, J. G., Meyer, H. E., Por, S. B., Smith, E., Williams, K. L. and Gooley, A. A. (1988). Structural characterization of *Dictyostelium discoideum* prespore specific gene D19 and of its product, cell surface glycoprotein PsA. *Mol. Cell. Biol.* **8**, 3458-3466.

Fisher, F. R., Smith, E. and Williams, K. L. (1981). An extracellular chemical signal controlling phototactic behavior by *D. discoideum* slugs. *Cell* **23**, 799-807.

Garcia, M. X. U., Foote, C., S., v. E., Devreotes, P. N., Alexander, S. and Alexander, H. (2000). Differential developmental expression and cell type specificity of *Dictyostelium* catalases and their response to oxidative stress and UV-light. *Biochim. Biophys. Acta* **1492**, 295-310.

Gooley, A. A., Marshchalek, R. and Williams, K. L. (1992). Size polymorphisms due to changes in the number of O-glycosylated tandem repeats in the *Dictyostelium discoideum* glycoprotein PsA. *Genetics* **130**, 749-756.

Gottlieb, D., Heideman, W. and Saba, J. D. (1999). The *DPL1* gene is involved in mediating the response to nutrient deprivation in *Saccharomyces cerevisiae*. *Mol. Cell. Biol. Res. Commun.* **1**, 66-71.

Igarashi, Y. (1997). Functional roles of sphingosine, sphingosine 1-phosphate, and methylsphingosines: in regard to membrane sphingolipid signaling pathways. *J. Biochem.* **122**, 1080-1087.

Kamakura, T., Kobayashi, K., Tanaka, T., Yamaguchi, I. and Endo, T. (1987). Cloning and expression of a new structural gene for blasticidin S deaminase, a nucleoside aminohydrolase. *Agric. Biol. Chem.* **51**, 3615-3618.

Kessin, R. H. (2001). Dictyostelium: *Evolution, Cell Biology and the Development of Multicellularity*. Cambridge, UK and New York: Cambridge University Press.

Kohama, T., Olivera, A., Edsall, L., Nagiec, M. M., Dickson, R. and Spiegel, S. (1998). Molecular cloning and functional characterization of murine sphingosine kinase. *J. Biol. Chem.* **273**, 23722-23728.

Kuspa, A. and Loomis, W. F. (1992). Tagging developmental genes in *Dictyostelium* by restriction enzyme-mediated integration of plasmid DNA. *Proc. Natl. Acad. Sci. USA* **89**, 8803-8807.

Laurence, A. and Firtel, R. (1999). Integration of signalling networks that regulate *Dictyostelium* differentiation. *Annu. Rev. Cell Dev. Biol.* **15**, 469-517.

Lee, S.-K., Yu, S.-L., Garcia, M. X., Alexander, H. and Alexander, S. (1997). Differential developmental expression of the *rep*B and *rep*D xeroderma pigmentosum related DNA helicase genes from *Dictyostelium discoideum*. *Nucleic Acids Res.* **25**, 2365-2374.

Li, G., Alexander, H., Schneider, N. and Alexander, S. (2000). Molecular basis for resistance to the anticancer drug cisplatin in *Dictyostelium*. *Microbiology* **146**, 2219-2227.

Loomis, W. F. (1998). Role of PKA in the timing of developmental events in *Dictyostelium* cells. *Microbiol. Mol. Biol. Rev.* **62**, 684-694.

Machwate, M., Rodan, S. B., Rodan, G. A. and Harada, S. I. (1998). Sphingosine kinase mediates cyclic AMP suppression of apoptosis in rat periosteal cells. *Mol. Pharmacol.* **54**, 70-77.

McRobbie, S. J., Jermyn, K. A., Duffy, K., Blight, K. and Williams, J. G. (1988). Two DIF inducible, prestalk specific messenger RNAs of *Dictyostelium* encode extracellular matrix proteins of the slug. *Development* **104**, 275-284.

Morio, T., Urushihara, H., Saito, T., Ugawa, Y., Mizuno, H., Yoshida, M., Yoshino, R., Mitra, B., Pi, M., Sato, T., Takemoto, K., Yasukawa, R., Williams, J., Maeda, M., Takeuchi, I. et al. (1998). The *Dictyostelium* developmental cDNA project: generation and analysis of expressed sequence tags from the first-finger stage of development. *DNA Res.* **5**, 335-340.

Muller, K. and Gerisch, G. (1978). A specific glycoprotein as the target site of adhesion blocking fab in aggregating *Dictyostelium* cells. *Nature* **274**, 445-449.

Newell, P. C., Telser, A. and Sussman, M. (1969). Alternative developmental pathways determined by environmental conditions in the cellular slime mold *Dictyostelium discoideum*. *J. Bacteriol.* **100**, 763-768.

Noegel, A. A., Rivero, F., Fucini, P., Bracco, E., Janssen, K. P. and Schleicher, M. (1997). Actin binding proteins: role and regulation. In Dictyostelium – *A Model System for Cell and Developmental Biology* (ed. Y. Maeda, K. Inouye and I. Takeuchi), pp. 33-42. Tokyo: Universal Academy Press.

Okamoto, H., Takuwa, N., Gonda, K., Okazaki, H., Chang, K., Yatomi, Y., Shigematsu, H. and Takuwa, Y. (1998). EDG1 is a functional sphingosine-1-phosphate receptor that is linked via a Gi/o to multiple signaling pathways, including phospholipase C activation, Ca2+ mobilization, Ras-mitogen-activated protein kinase activation, and adenylate cyclase inhibition. *J. Biol. Chem.* **273**, 27104-27110.

Parent, C. A. and Devreotes, P. N. (1999). A cell's sense of direction. *Science* **284**, 765-770.

Pyne, S. and Pyne, N. J. (1996). The differential regulation of cyclic AMP by sphingomyelin-derived lipids and the modulation of sphingolipid-stimulated extracellular signal regulated kinase-2 in airway smooth muscle. *Biochem. J.* **315**, 917-923.

Pyne, S. and Pyne, N. J. (2000). Sphingosine 1-phosphate signalling in mammalian cells. *Biochem. J.* **349**, 385-402.

Rani, C. S., Wang, F., Fuior, E., Berger, A., Wu, J., Sturgill, T. W., Beitner-Johnson, D., LeRoith, D., Varticovski, L. and Spiegel, S. (1997). Divergence in signal transduction pathways of platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptors. Involvement of sphingosine 1-phosphate in PDGF but not EGF signaling. *J. Biol. Chem.* **272**, 10777-10783.

Richardson, D. L. and Loomis, W. F. (1992). Disruption of the sporulation-specific gene *spi*A in *Dictyostelium discoideum* leads to spore instability. *Genes Dev.* **6**, 1058-1070.

Saba, J. D., Nara, F., Bielawska, A., Garrett, S. and Hannun, Y. A. (1997). The BST1 gene of *Saccharomyces cerevisiae* is the sphingosine-1-phosphate lyase. *J. Biol. Chem.* **272**, 26087-26090.

Sadahira, Y., Ruan, F., Hakomori, S. and Igarashi, Y. (1992). Sphingosine 1-phosphate, a specific endogenous signaling molecule controlling cell motility and tumor cell invasiveness. *Proc. Natl. Acad. Sci. USA* **89**, 9686-9690.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sliva, D., Mason, R., Xiao, H. and English, D. (2000). Enhancement of the migration of metastatic human breast cancer cells by phosphatidic acid. *Biochem. Biophys. Res. Commun.* **268**, 471-479.

Soll, D. (1987). Methods for manipulating and investigating developmental timing in *Dictyostelium discoideum*. *Methods Cell Biol.* **28**, 413-431.

Spiegel, S. (1999). Sphingosine 1-phosphate: a prototype of a new class of second messengers. *J. Leukoc. Biol.* **65**, 341-344.

Spiegel, S. and Milstien, S. (2000a). Functions of a new family of sphingosine-1-phosphate receptors. *Biochim. Biophys. Acta* **1484**, 107-116.

Spiegel, S. and Milstien, S. (2000b). Sphingosine-1-phosphate: signaling inside and out. *FEBS Lett.* **476**, 55-57.

Srinivasan, S., Griffiths, K. R., McGuire, V., Champion, A., Williams, K. L. and Alexander, S. (2000). The cellulose-binding activity of the PsB multiprotein complex is required for proper assembly of the spore coat and spore viability in Dictyostelium discoideum. *Microbiology* **146**, 1829-1839.

Sussman, M. (1987). Cultivation and synchronous morphogenesis of *Dictyostelium* under controlled experimental conditions. *Methods Cell Biol.* **28**, 9-29.

Sussman, M. and Brackenbury, R. (1976). Biochemical and molecular-genetic aspects of cellular slime mold development. *Annu. Rev. Plant Physiol.* **27**, 229-265.

Tolan, D., Conway, A. M., Pyne, N. J. and Pyne, S. (1997). Sphingosine prevents diacylglycerol signaling to mitogen-activated protein kinase in airway smooth muscle. *Am. J. Physiol.* **273**, C928-C936.

Van Brocklyn, J. R., Lee, M. J., Menzeleev, R., Olivera, A., Edsall, L., Cuvillier, O., Thomas, D. M., Coopman, P. J., Thangada, S., Liu, C. H., Hla, T. and Spiegel, S. (1998). Dual actions of sphingosine-1-phosphate: extracellular through the Gi- coupled receptor Edg-1 and intracellular to regulate proliferation and survival. *J. Cell Biol.* **142**, 229-240.

van Veldhoven, P. P. and Mannaerts, G. P. (1993). Sphingosine-phosphate lyase. *Adv. Lipid Res.* **26**, 69-98.

Wang, F., Nohara, K., Olivera, A., Thompson, E. W. and Spiegel, S. (1999a). Involvement of focal adhesion kinase in inhibition of motility of human breast cancer cells by sphingosine 1-phosphate. *Exp. Cell Res.* **247**, 17-28.

Wang, F., Van Brocklyn, J. R., Edsall, L., Nava, V. E. and Spiegel, S. (1999b). Sphingosine-1-phosphate inhibits motility of human breast cancer cells independently of cell surface receptors. *Cancer Res.* **59**, 6185-6191.

Wessels, D. and Soll, D. R. (1998). Computer-assisted characterization of the behavioral defects of cytoskeletal mutants of *Dictyostelium discoideum*. In *Motion Analysis of Living Cells* (ed. D. R. Soll and D. Wessels), pp. 101-140. New York: Wiley-Liss.

Wu, J., Spiegel, S. and Sturgill, T. W. (1995). Sphingosine-1-phosphate rapidly activates the mitogen-activated protein kinase by a G protein-dependent mechanism. *J. Biol. Chem.* **270**, 11484-11488.

Yamamura, S., Sadahira, Y., Ruan, F., Hakomori, S. and Igarashi, Y. (1996). Sphingosine-1-phosphate inhibits actin nucleation and pseudopodium formation to control cell motility of mouse melanoma cells. *FEBS Lett.* **382**, 193-197.

Yamamura, S., Yatomi, Y., Ruan, F., Sweeney, E. A., Hakomori, S. and Igarashi, Y. (1997). Sphingosine 1-phosphate regulates melanoma cell motility through a receptor-coupled extracellular action and in a pertussis toxin- insensitive manner. *Biochemistry* **36**, 10751-10759.

Yatomi, Y., Yamamura, S., Ruan, F. and Igarashi, Y. (1997). Sphingosine 1-phosphate induces platelet activation through an extracellular action and shares a platelet surface receptor with lysophosphatidic acid. *J. Biol. Chem.* **272**, 5291-5297.

Zada-Hames, I. M. and Ashworth, J. M. (1978). The cell cycle and its relationship to development in Dictyostelium discoideum. *Dev. Biol.* **63**, 307-320.