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Differential transactivation of sphingosine-1-phosphate receptors modulates NGF-induced neurite extension

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The process of neurite extension after activation of the TrkA tyrosine kinase receptor by nerve growth factor (NGF) involves complex signaling pathways. Stimulation of sphingosine kinase 1 (SphK1), the enzyme that phosphorylates sphingosine to form sphingosine-1-phosphate (S1P), is part of the functional TrkA signaling repertoire. In this paper, we report that in PC12 cells and dorsal root ganglion neurons, NGF translocates SphK1 to the plasma membrane and differentially activates the S1P receptors $S1P_1$ and $S1P_2$ in a SphK1-dependent manner, as determined with specific inhibitors and small interfering RNA targeted to SphK1. NGF-induced neurite extension was suppressed by down-regulation of $S1P_1$ expression with antisense RNA. Conversely, when overexpressed in PC12 cells, transactivation of $S1P_1$ by NGF markedly enhanced neurite extension and stimulation of the small GTPase Rac, important for the cytoskeletal changes required for neurite extension. Concomitantly, differentiation down-regulated expression of $S1P_2$ whose activation would stimulate Rho and inhibit neurite extension. Thus, differential transactivation of S1P receptors by NGF regulates antagonistic signaling pathways that modulate neurite extension.

Introduction

The archetypal neurotrophin NGF has long been recognized for its roles in the survival, differentiation, and target innervation by sympathetic and sensory neurons, as well as the elaboration of neurites in rat pheochromocytoma PC12 cells, a model for sympathetic neurons. Signaling through the TrkA receptor tyrosine kinase is central to NGF action. Dimerization and autophosphorylation of TrkA couples this receptor to intracellular signaling cascades, including the Ras/ERK, phosphoinositide 3-kinase (PI3K)/Akt, and phospholipase Cγ pathways (for review see Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001).

In addition to well-established TrkA signaling systems, we previously found that TrkA activates sphingosine kinase type 1 (SphK1; Edsall et al., 1997), the enzyme that phosphorylates sphingosine to form sphingosine-1-phosphate (S1P). S1P, an important lipid mediator (Spiegel and Milstien, 2002), is the ligand for five specific G protein–coupled receptors (GPCRs), designated $S1P_1$–$S1P_5$. These receptors are enriched in the nervous system and couple to different G proteins to regulate diverse signaling cascades. In particular, $S1P_1$, $S1P_2$, and $S1P_5$ are highly expressed and temporally regulated in the brain (Lado et al., 1994; Glickman et al., 1999). $S1P_2$ expression is highest in neuronal cell bodies during early stages of differentiation and in axons during branching (MacLennan et al., 1997). Moreover, during NGF-induced differentiation of PC12 cells, which endogenously express $S1P_1$ (Lado et al., 1994), $S1P_2$ (MacLennan et al., 2000), $S1P_3$ (Molderings et al., 2002), and $S1P_5$ (Glickman et al., 1999), expression of $S1P_2$ and $S1P_5$, but

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Abbreviations used in this paper: DMS, *N*,*N*-dimethylsphingosine; DRG, dorsal root ganglion; GPCR, G protein–coupled receptor; PI3K, phosphoinositide 3-kinase; PTX, pertussis toxin; S1P, sphingosine-1-phosphate; siRNA, small interfering RNA; SphK, sphingosine kinase.

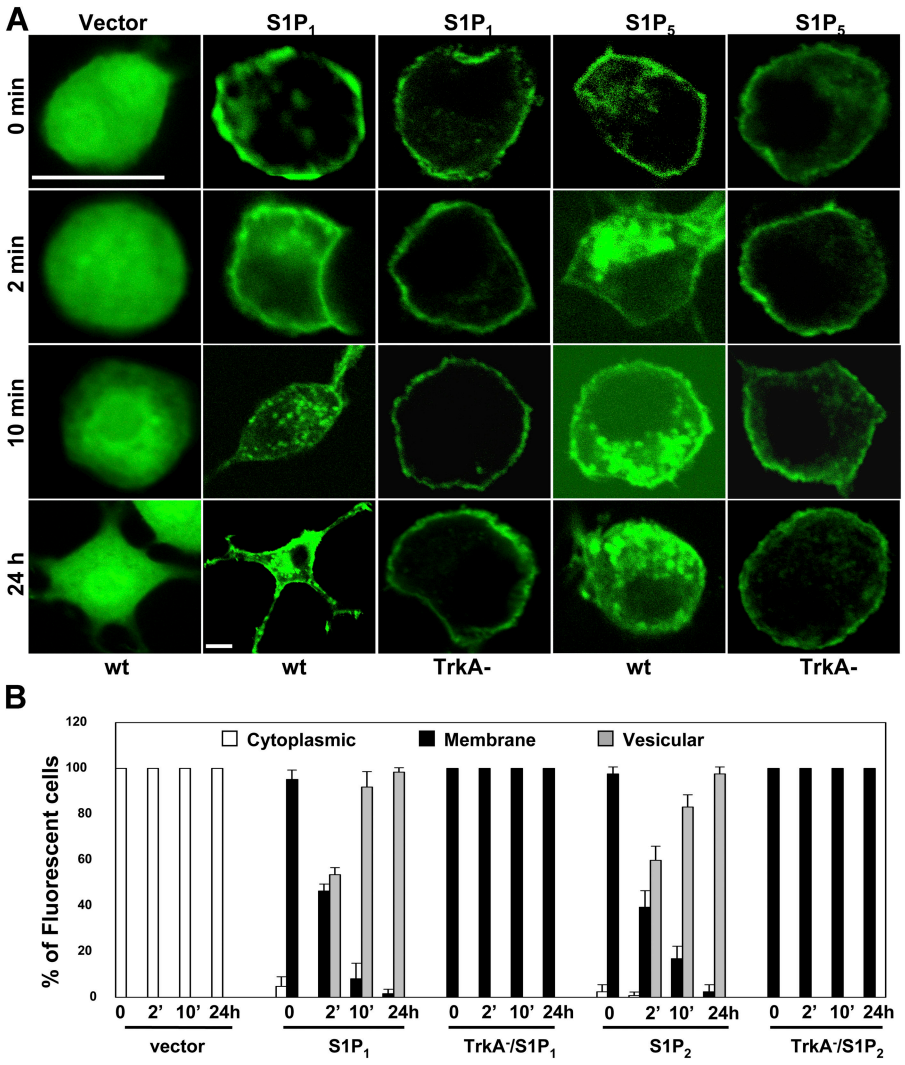

Figure 1. **NGF-induced S1P receptor internalization is TrkA dependent.** (A and B) Naive or TrkA-less nnr5 PC12 cells were transfected with expression plasmids encoding GFP, $S1P_1$-GFP, or $S1P_5$-GFP and were cultured for 48 h. Serum-starved cells were treated with 100 ng/ml NGF for the indicated times and were visualized by confocal microscopy. (A) Representative cells of more than 100 cells examined are shown. (B) S1P receptor localization was quantified by assessing the percentage of GFP-positive cells with cytoplasmic staining (open bars, GFP was present inside the cell without any indication of discrete structural staining), membrane staining (black bars, GFP outlined the cell with little if any GFP present inside the cell), or vesicular staining (gray bars, GFP was present inside the cell in discrete, punctate patterns). The absence of bars indicates none detected. Results are means ± SD from four independent experiments. At least 100 cells per condition were counted. Bars, 25 μm.

not $S1P_1$, has been reported to be down-regulated (Glickman et al., 1999). Although the biological functions of these S1P receptors in the nervous system are largely unknown, they all have been implicated in directed cell movement and cytoskeleton rearrangements (Spiegel and Milstien, 2002).

Neurite extension and retraction, important processes in the establishment of neuronal networks during development, are largely orchestrated by the organization of the actin cytoskeleton controlled by the balance between the opposing actions of the small GTPases, Rho and Rac (Li et al., 2002). Although Rho induces collapse of growth cones and inhibition of neurite outgrowth (Nakamura et al., 2002), Rac is required for neurite outgrowth (Estrach et al., 2002). Of interest, in diverse cell types, S1P receptors differentially regulate Rac and Rho. Binding of S1P to $S1P_1$ enhances Rac-coupled cortical actin formation (Rosenfeldt et al., 2001), whereas $S1P_2$ stimulates Rho and suppresses activation of Rac, thereby inhibiting cell migration (Okamoto et al., 2000).

In this work, we examined the role of S1P receptors in NGF-induced neurite extension. We found that NGF, in a TrkA-dependent manner, transactivated S1P receptors in PC12 cells by stimulation of SphK1. Neurite elongation of cultured dorsal root ganglion (DRG) neurons induced by NGF was similarly regulated via SphK1 and activation of S1P receptors. Our results reveal that cross-communication between TrkA and S1P receptors, and the consequent regulation of opposing signaling pathways, such as Rac and Rho, may play an important role in neurite extension.

Results

NGF activates S1P receptors in PC12 cells

Similar to other GPCRs, GFP-tagged S1P receptors have been extensively used to study receptor internalization after activation and have been shown to transduce intracellular signals in a manner indistinguishable from the wild-type receptors (Liu et al., 1999). As expected, $S1P_1$-GFP and $S1P_5$-GFP were expressed mainly on the plasma membrane of serum-starved PC12 cells while GFP-vector was cytoplasmic (Fig. 1 A). Interestingly, internalization of $S1P_1$ and $S1P_5$ was significant within 2 min after addition of NGF (Fig. 1 A). The internalization occurred in the absence of exogenous S1P and was clearly evident within 10 min (Fig. 1, A and B). This time course is similar to that of S1P-induced trafficking of $S1P_1$ in HEK 293 cells (Liu et al., 1999). On the other hand, NGF did not induce internalization of $S1P_1$ or $S1P_5$

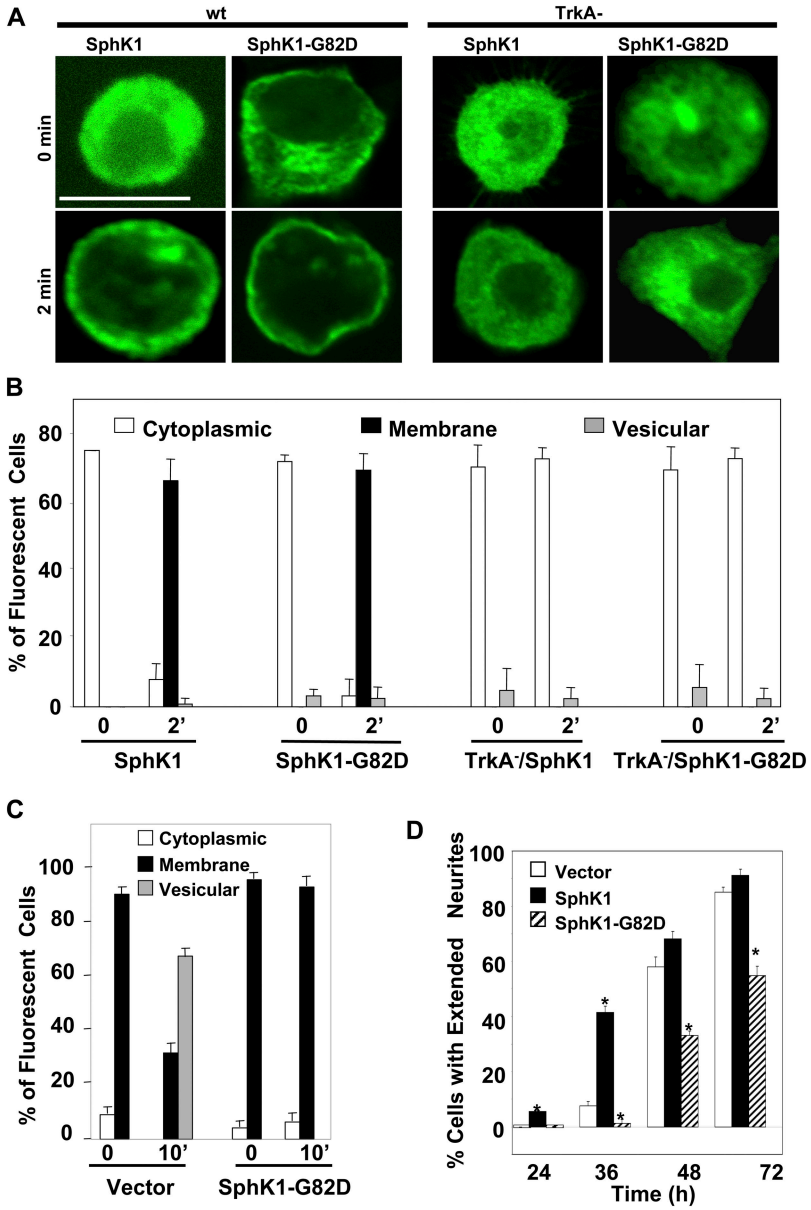

Figure 2. **NGF-induced translocation of SphK1.** (A and B) Wild-type or nnr5 PC12 cells were transfected with either SphK1-GFP or catalytically inactive SphK1-G82D-GFP and were cultured for 48 h. Serum-starved cells were treated without or with NGF (100 ng/ml) for 2 min and were visualized by confocal microscopy. (A) Representative cells of more than 100 cells examined are shown. Bar, 25 μm. (B) The percentage of GFP-positive cells with cytosolic staining (open bars), membrane staining (black bars), or vesicular staining (gray bars) were enumerated in a blinded manner. The absence of bars indicates none detected. Results are means ± SD from three independent experiments. (C) PC12 cells were cotransfected with vector or catalytically inactive SphK1-G82D and $S1P_1$-GFP and were cultured for 48 h. Serum-starved cells were treated without or with NGF (100 ng/ml) for 10 min, visualized by confocal microscopy, and quantified as described in B. (D) SphK1 is critical for neurite extension. Wild-type PC12 cells were transfected with vector-GFP, SphK1-GFP, or SphK1-G82D-GFP and were cultured for 48 h. Serum-starved cells were treated without or with NGF (100 ng/ml) for the indicated times and examined by fluorescence microscopy. Neurite extension was quantified by assessing the percentage of GFP-positive cells bearing at least one neurite twice the length of the cell body. Asterisks denote significant differences relative to vector transfected cells ($P < 0.01$, ANOVA, Tukey's).

in nnr5 cells, a TrkA-deficient PC12 mutant that is unresponsive to NGF-induced neuritogenesis (Loeb et al., 1991) (Fig. 1, A and B). These results suggest that NGF transactivates $S1P_1$ and $S1P_5$ in a TrkA-dependent manner.

SphK1 is required for NGF-induced transactivation of S1P receptors

Previously, we have shown that binding of NGF to TrkA stimulates SphK1, leading to a rapid increase in S1P levels in PC12 cells (Edsall et al., 1997). Although SphK1 is diffusely distributed in the cytosol of unstimulated PC12 cells, as it is in other cell types (Olivera et al., 1999), NGF induced its rapid translocation to the plasma membrane where its substrate sphingosine resides (Fig. 2, A and B). In sharp contrast, SphK1 appeared to be entirely cytoplasmic in nnr5 cells and translocation to the plasma membrane by NGF was not evident (Fig. 2, A and B), even after much longer exposure (unpublished data).

To examine whether SphK1 activity is required for NGF-induced translocation of SphK1 and S1P receptors, we used a catalytically inactive, dominant-negative mutant SphK1 prepared by site-directed mutagenesis of the second conserved glycine residue in the ATP-binding site ($SGDGX_{17-21}K(R)$) present within the conserved catalytic domain of all SphK isoforms. In untreated PC12 cells, the catalytically inactive G82D mutant (SphK1-G82D) had a similar diffuse cytosplasmic distribution as wild-type SphK1. NGF treatment also translocated SphK1-G82D to the plasma membrane in a TrkA-dependent manner (Fig. 2, A and B), and the catalytically inactive mutant prevented NGF-induced internalization of $S1P_1$ (Fig. 2 C).

In PC12 cells, NGF initiates time-dependent cellular differentiation and neurite outgrowth (Greene and Tischler, 1976). Because NGF translocates (Fig. 2 A) and stimulates SphK1 (Edsall et al., 1997), it was of interest to examine the role of SphK1 in neuritogenesis. In both naive and vector-

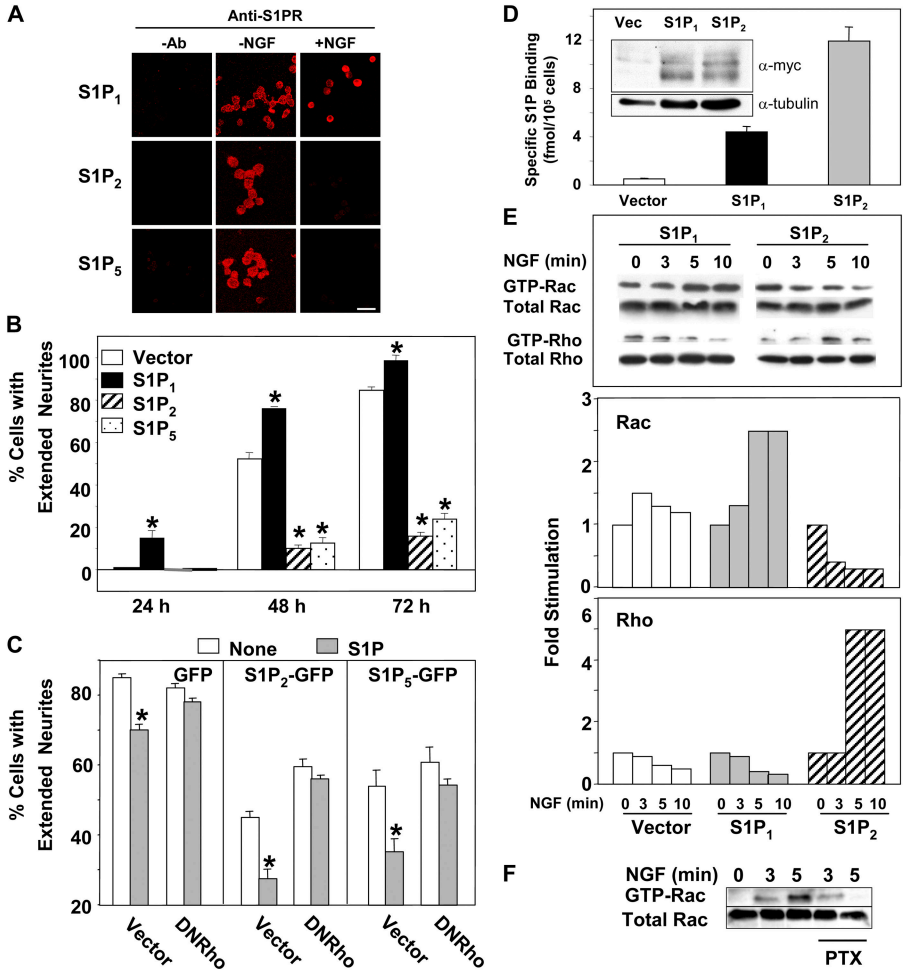

Figure 3. **Activation of $S1P_1$ stimulates, whereas $S1P_2$ and $S1P_5$ inhibit neurite extension.** (A) PC12 cells were plated on poly-D-lysine–coated coverslips and cultured in the absence (−) or presence (+) of NGF (100 ng/ml) for 24 h. Immunocytochemical analysis of S1P receptor expression was performed with specific antibodies as indicated and was visualized by rhodamine-conjugated secondary antibody. No appreciable background staining was evident with IgG or when primary antibody was omitted or preincubated with the peptide antigen. Bar, 100 μm. (B) PC12 cells were transfected with vector-GFP (open bars), $S1P_1$-GFP (filled bars), $S1P_2$-GFP (hatched bars), or $S1P_5$-GFP (stippled bars) and were cultured for 48 h. Serum-starved cells were treated with 100 ng/ml NGF for the indicated times and neurite extension was quantified by assessing the percentage of GFP-positive cells bearing at least one neurite twice the length of the cell body. Asterisks denote significant differences relative to vector transfectants ($P < 0.01$, ANOVA, Tukey's). (C) PC12 cells were cotransfected with vector or N19RhoA (DNRho) together with vector-GFP, $S1P_2$-GFP, or $S1P_5$-GFP, as indicated, at a ratio of 5:1 to ensure that GFP-expressing cells also express DNRho. Serum-starved cells were incubated with 100 ng/ml NGF for 120 h and treated without (open bars) or with S1P (100 nM; gray bars) for the last 3 h. Neurite extension was quantified by assessing the percentage of GFP-positive cells bearing at least one neurite twice the length of the cell body. Asterisks denote significant differences relative to untreated cells ($P < 0.01$, ANOVA, Tukey's). (D) Specific binding of [^{32}P]S1P (defined as binding in the absence of unlabeled competitor minus binding in the presence of excess unlabeled ligand) to PC12 cells stably expressing vector, myc-$S1P_1$, or myc-$S1P_2$ was measured as described previously (Van Brocklyn et al., 1999). Inset: Western blot of cell lysates was probed with anti-Myc or anti-tubulin antibodies (Santa Cruz Biotechnology, Inc.) as described previously (Watterson et al., 2002). (E) Serum-starved PC12 cells stably transfected with vector (open bars), myc-$S1P_1$ (gray bars), or myc-$S1P_2$ (hatched bars) were stimulated with 100 ng/ml NGF for the indicated times. Activated Rac and Rho were specifically pulled down from cell lysates containing equal amounts of proteins and were analyzed by Western blotting using anti-Rac and anti-Rho antibodies, respectively. Relative activated levels were normalized to total Rac and Rho and the fold stimulation is depicted. Representative results from one of three independent experiments are shown. (F) Wild-type PC12 cells were cultured in serum-free medium without or with PTX (100 ng/ml) for 16 h, then stimulated with NGF for the indicated times and Rac activation determined.

transfected PC12 cells, there was no detectable neurite extension 1 d after NGF treatment and only minimal extension by 36 h (Fig. 2 D). In contrast, SphK1 overexpression enhanced the initiation of NGF-induced PC12 cell differentiation, resulting in a significant increase in PC12 cells bearing neurites twice the length of their cell bodies that was evident within 1 d and marked at 36 h. However, at 48 h, vector- and SphK1-transfected cells exhibited similarly extended neurites. Moreover, expression of SphK1-G82D significantly inhibited the rate of neurite extension induced by NGF (Fig. 2 D). These results suggest that SphK1 might play a role in NGF-mediated neurite elaboration.

Transactivation of S1P receptors by NGF differentially affects neurite extension

Untreated naive PC12 cells endogenously express mRNA for $S1P_1$ (Lado et al., 1994), $S1P_2$ (MacLennan et al., 2000), $S1P_3$ (Molderings et al., 2002), and $S1P_5$ (Glickman et al., 1999). A recent analysis established the successful use of anti-S1P receptor antibodies for immunocytochemical analysis in diverse cell types (Jin et al., 2003). Immunohistochemistry with specific S1P receptor antibodies also revealed the expression of $S1P_1$, $S1P_2$, and $S1P_5$ in PC12 cells (Fig. 3 A). In agreement with previous reports (Lado et al., 1994; Glickman et al., 1999; MacLennan et al., 2000), treatment with NGF down-regulated all of the S1P receptors except $S1P_1$ as demonstrated by RT-PCR (unpublished data) and by the nearly complete disappearance of immunohistochemical labeling with anti-$S1P_2$ and anti-$S1P_5$, but not with anti-$S1P_1$ (Fig. 3 A).

Next, we examined the involvement of S1P receptors in neurite extension. Overexpression of $S1P_1$ remarkably accelerated and strongly potentiated NGF-induced neurite extension in these cells, again in the absence of exogenous S1P. Within

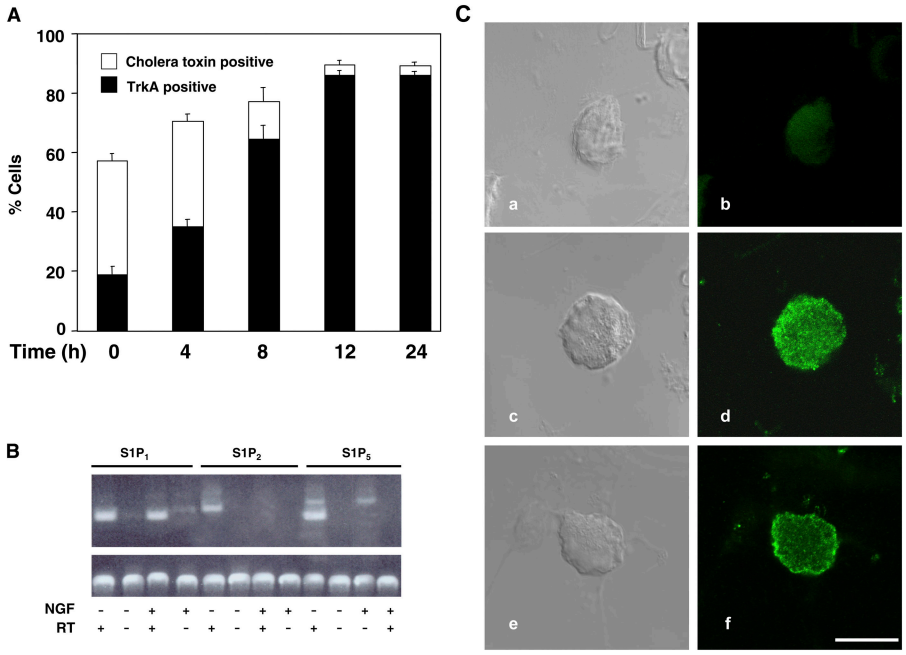

Figure 4. **Expression of S1P receptors in DRG neurons.** (A) Rat E15 DRG neurons were dissociated, plated on growth factor–reduced Matrigel™-coated coverslips and cultured in 100 ng/ml NGF-containing medium for the indicated times. Fixed cells were stained with cholera toxin B/Alexa Fluor®, costained with TrkA-specific antibody, and visualized with rhodamine-conjugated secondary antibody. The percentage of neurons, indicated by the number of cholera toxin–positive cells (white bars), and the percentage of TrkA-positive neurons (filled bars), were quantitated. Each point is the mean ± SD of triplicates. A total of at least 300 cells were scored. The data are representative of two independent experiments. (B) RT-PCR analysis of $S1P_1$, $S1P_2$, and $S1P_5$ mRNA expression in DRG neurons 30 min after plating (−) or 16 h (+) in the presence of 100 ng/ml NGF. PCR reactions were performed without (−) or with (+) reverse transcription. GAPDH mRNA expression was determined as a control. (C) NGF induces translocation of endogenous SphK1 in DRG neurons. Rat E15 DRG neurons were dissociated, plated on growth factor–reduced Matrigel™-coated coverslips and cultured in chemically defined medium for 6 h. Cells were then treated without (a–d) or with (e and f) NGF (100 ng/ml) for 10 min, fixed, and stained without (a and b) or with (c–f) SphK1-specific antibody, and were visualized with Cy2-conjugated secondary antibody. Cells were examined by confocal fluorescence microscopy (b, d, and f) and differential interference contrast microscopy (a, c, and e). No significant fluorescence was detected when the primary antibody was omitted (b). Representative cells from more than 20 examined are shown. Bar, 25 μm.

1 d, nearly 20% of these cells had neurites twice the length of their cell bodies (Fig. 3 B), whereas barely detectable neurite extension was observed in naive or vector-transfected PC12 cells, and no neurites were detectable in $S1P_2$- or $S1P_5$-expressing cells (Fig. 3 B). This dramatic stimulation of NGF-induced neurite formation at 24 h was also clearly evident in the morphology of $S1P_1$-transfected PC12 cells (Fig. 1 A, bottom). Moreover, overexpression of $S1P_1$ enhanced NGF-induced differentiation even after prolonged exposure. By contrast, overexpression of either $S1P_2$ or $S1P_5$ strongly inhibited NGF-induced neurite extension (Fig. 3 B). Similar results were obtained with PC12 cells that were transfected with wild-type $S1P_1$, $S1P_2$, or $S1P_5$, together with GFP at a 5:1 ratio to identify transfected cells (unpublished data), further supporting the notion that the GFP tag does not interfere with functions of the S1P receptors (Liu et al., 1999). When S1P was added to PC12 cells overexpressing $S1P_2$ or $S1P_5$ for only the final 3 h of a 120-h culture period in the presence of NGF, it initiated retraction of existing neurites (Fig. 3 C). Similarly, wild-type $S1P_2$ and $S1P_3$, but not $S1P_1$, have previously been associated with S1P-induced rounding of NGF-differentiated PC12 cells (Van Brocklyn et al., 1999).

The opposing roles of Rac and Rho in neurite extension modulated by S1P receptors

The Rho family of GTPases, Rho, Rac, and Cdc42, has been shown to play an important role in neuronal morphogenesis and differentiation (Li et al., 2002; Nusser et al., 2002). In particular, Rho mediates retraction of neurites and inhibits their outgrowth (Yamaguchi et al., 2001; Nakamura et al., 2002; Nusser et al., 2002). Thus, we first examined the role of Rho in $S1P_2$- and $S1P_5$-mediated suppression of neuritogenesis by expressing a dominant-negative mutant Rho (N19RhoA). This mutant Rho inhibited the effect of S1P on neurite retraction mediated by both $S1P_2$ and $S1P_5$ (Fig. 3 C), in agreement with previous reports showing that S1P-induced neurite retraction and soma rounding of N1E-115 neuronal cells was blocked by *Clostridium botulinum* C3 transferase, which ADP-ribosylates and inactivates Rho (Postma et al., 1996). Expression of dominant-negative N17Rac resulted in nearly complete suppression of neuritogenesis in both vector and S1P receptor transfected cells, highlighting the critical importance of Rac in the process of neurite outgrowth.

Because it is well established that $S1P_1$ and $S1P_2$ can differentially regulate Rho and Rac, it was of interest to assess the roles of the S1P receptors in modulation of activation of Rho and Rac induced by NGF in PC12 cells. To this end, we measured the levels of active, GTP-bound forms of Rac and Rho by GST pull-down assays with the GST-fused CRIB-containing NH_2 terminus binding domain of PAK and the GST-fused binding domain of rhotekin, respectively. To increase the sensitivity, we used PC12 cells stably expressing $S1P_1$ or $S1P_2$ as described previously (Van Brocklyn et al., 1999). Nonclonal pools were used to avoid potential phenotypic changes due to selection and propagation of clones derived from single individual cells. Overexpression of $S1P_1$ or $S1P_2$ resulted in marked increases in specific S1P binding as expected (Fig. 3 D). Similar to previous reports (Yamaguchi et al., 2001; Nusser et al., 2002), NGF rapidly and transiently increased GTP-Rac and reduced GTP-Rho (Fig. 3 E). In agreement with the opposing effects of differ-

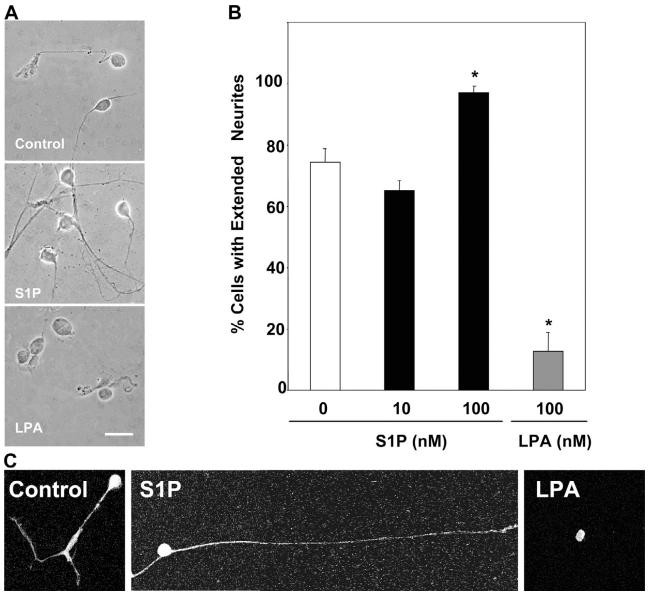

Figure 5. **S1P accelerates neurite extension induced by NGF in DRG neurons.** Rat E15 DRG neurons were dissociated, plated on growth factor–reduced Matrigel™-coated coverslips, and exposed to 100 nM S1P or lysophosphatidic acid (LPA) for the final 90 min of a 16-h post-dissection culture period in 100 ng/ml NGF-containing medium. Photomicrographs of representative neurons were examined by phase microscopy (A) or stained with rhodamine-phalloidin to visualize actin filaments, and were examined by fluorescence microscopy (C). (B) Neurite extension was quantified by assessing the percentage of neurons bearing at least one neurite four times the length of the cell body. Asterisks denote significant differences relative to untreated cells ($P < 0.01$, ANOVA, Tukey's). Bar, 50 μm.

ent S1P receptors on neurite extension (Fig. 3 B), overexpression of $S1P_1$ enhanced NGF-induced Rac activation, whereas NGF treatment of $S1P_2$-expressing PC12 cells inhibited Rac activation and stimulated Rho (Fig. 3 E). To further examine the involvement of $S1P_1$ in NGF-induced Rac activation, PC12 cells were pretreated with pertussis toxin (PTX) to inactivate G_i, as $S1P_1$, in contrast to the other S1P receptors, is coupled solely to G_i. PTX reduced NGF-induced Rac activation (Fig. 3 F). Similarly, NGF-induced differentiation of PC12 cells is also sensitive to PTX (Rakhit et al., 2001).

S1P modulates NGF-induced differentiation of rat DRG neurons

To investigate the role of S1P and S1P receptors in regulation of neurite extension in primary neurons, we used DRG neurons isolated from embryonic rats. Not only do these neurons express TrkA, they have also been shown to respond to NGF (Markus et al., 2002a). To eliminate unintended lipid effects, the dissociated DRG neurons were grown in the absence of serum with NGF acting as the sole trophic factor. Although only 30% of the freshly dissociated neurons were TrkA positive, in agreement with some reports (Mu et al., 1993; Kashiba et al., 1995), other analyses using stained tissue sections or RNA hybridization assays (Wright and Snider, 1995; Molliver and Snider, 1997) have found higher proportions of TrkA-expressing cells. This variability could result from the trypsinization treatment necessary to dissociate the ganglia. Nevertheless, culturing the DRG neurons in the presence of NGF resulted in the death of most of the neurons not expressing TrkA, producing nearly homogenous TrkA-expressing cultures by 16 h (Fig. 4 A). Freshly dissociated DRG neurons express mRNA for $S1P_1$, $S1P_2$, and $S1P_5$ (Fig. 4 B), indicating the potential for endogenous S1P signaling. Similar to PC12 cells, culturing these neurons in NGF-containing media resulted in the down-regulation of both $S1P_2$ and $S1P_5$, but not $S1P_1$ (Fig. 4 B).

Because NGF induced translocation of SphK1 in PC12 cells, we next examined its effect on localization of endogenous SphK1 in DRG neurons. We used a specific antibody that recognizes SphK1 in rat brain and shows a single band of the expected size of 46 kD on immunoblots (Murate et al., 2001). Confocal immunohistochemistry revealed that similar to SphK1 overexpressing PC12 cells, endogenous SphK1 was mainly cytosolic in unstimulated DRG neurons (Fig. 4 C, d). NGF treatment induced translocation of SphK1 to the plasma membrane within 10 min (Fig. 4 C, f). This is consistent with movement of SphK1 to the plasma membrane by other agonists in different cell types (Rosenfeldt et al., 2001; Johnson et al., 2002).

The majority of freshly dissociated DRGs were rounded and had no processes before treatment with NGF. NGF induced a time-dependent increase in the number of neurons extending processes, and within 16 h, >70% of DRG neurons had extensive neurites of at least four times the cell body length (Fig. 5). Addition of 100 nM S1P for the last 1.5 h markedly potentiated neurite extension, increasing the number of neurons with neurites to nearly 100% (Fig. 5 B). Moreover, S1P-treated neurons had significantly elongated neurites that tended to have greater distal branching (Fig. 5, A and C). This was a specific response to S1P, as lysophosphatidic acid, a structurally related lysophospholipid that binds to a related yet distinct GPCR family, caused neurite retraction rather than extension at similar concentrations (Fig. 5).

Surprisingly, during the course of this investigation we noticed that when dissociated DRG neurons were exposed to both NGF and S1P at the time of plating, S1P inhibited neurite extension in a concentration-dependent manner, with a significant effect at a concentration as low as 10 nM and maximal inhibition at 100 nM (Fig. 6, A and B). S1P abrogated NGF-stimulated axonal growth without killing the cells. The striking contrast in responses to S1P at different stages of neurite development is reminiscent of its effects on PC12 cells and suggests that S1P and its receptors might exhibit temporally specific functions. Given that the opposing effect of S1P on neurite extension and retraction in PC12 cells was dependent on relative expression of the various S1P receptors, it was important to further substantiate that their levels in DRG neurons varied with the extent of neurite formation. In agreement with mRNA analyses (Fig. 4 B), immediately after dissociation, E15 DRG neurons expressed both $S1P_1$ and $S1P_2$ as determined by immunocytochemistry (Fig. 6 C). After culturing for 4 h in media containing NGF, DRG neurons expressed only $S1P_1$, which was expressed on the cell surface (Fig. 6 C). After 16 h of NGF treatment, $S1P_1$ was expressed in the soma as well as along the extended neurites (Fig. 6 D). These results suggest that

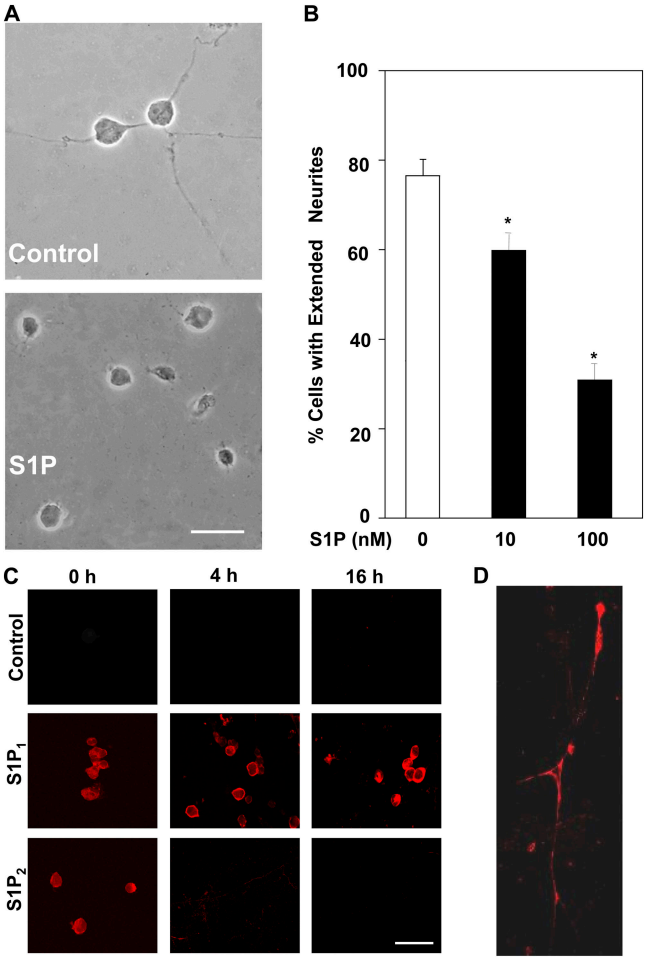

Figure 6. **S1P suppresses NGF-induced neurite extension in freshly dissociated DRG neurons.** (A) Rat E15 DRG neurons were dissociated, plated on growth factor–reduced Matrigel™-coated coverslips and treated without or with 100 nM S1P (A) or the indicated concentrations of S1P (B) in 100 ng/ml NGF-containing medium. Photomicrographs of representative neurons examined by phase microscopy are shown. (B) Neurite extension was quantified after 16 h by assessing the percentage of neurons bearing at least one neurite four times the length of the cell body. A minimum of 300 neurons was examined and data are the averages ± SD. Similar results were obtained in three independent experiments. Asterisks denote significant differences relative to untreated cells ($P < 0.01$, ANOVA, Tukey's). (C) Differential expression of S1P receptors during development of DRG neurons. Rat E15 DRG neurons were dissociated, plated on growth factor–reduced Matrigel™-coated coverslips, and cultured in the presence of NGF for 0, 4, and 16 h. Immunocytochemical analysis of S1P receptor expression was performed with specific antibodies as indicated and stained with rhodamine-conjugated secondary antibody and visualized by fluorescence microscopy. No appreciable background staining was evident with IgG or when primary antibody (control) was omitted or preincubated with the peptide antigen. (D) Confocal collage of a representative neuron treated with NGF for 16 h and stained with anti-$S1P_1$ showing expression of $S1P_1$ on extended neurites. Bars: 50 μm (A); 100 μm (C).

similar to PC12 cells, $S1P_2$ protein and mRNA expression is down-regulated in DRG neurons during neurite elongation.

Role of $S1P_1$ in neurite extension of DRG neurons

Because $S1P_1$ is prominently expressed on DRG neurons during neurite extension and is coupled to G_i, we used PTX to examine the role of $S1P_1$ in this process. When NGF-treated, neurite-possessing DRG neurons were pretreated with PTX 3 h before adding S1P, PTX completely suppressed neurite extension induced by S1P (Fig. 7 A). To further confirm the role of $S1P_1$ in S1P-induced DRG neurite extension, $S1P_1$ expression was down-regulated by transfection with $S1P_1$ antisense RNA, which was designed to bind to the translation initiation site on the $S1P_1$ mRNA and thus block its expression. Previously, we have shown that $S1P_1$ mRNA expression was almost completely eliminated in aortic smooth muscle cells by $S1P_1$ antisense oligonucleotides without affecting $S1P_2$ or $S1P_3$ expression (Hobson et al., 2001). Antisense oligonucleotides for $S1P_1$ specifically inhibited endogenous neurite extension, as sense and scrambled oligonucleotides had no effects (Fig. 7, B and C). To confirm that the neurons whose S1P receptors are repressed by antisense are those that have shorter neurites, fluorescent oligonucleotides were used in these experiments and effects on neuritogenesis of fluorescent neurons compared in the same cultures (Fig. 7 C) to the nonfluorescent (Fig. 7 D) and therefore nontransfected neurons. Importantly, nontransfected neurons in the same cultures behaved similarly as neurons transfected with the sense and scrambled oligonucleotides (Fig. 7, C and D). Not only did antisense $S1P_1$ oligonucleotide significantly decrease the number of neurons with extensive neurites, nearly 31% of these cells failed to exhibit any neurite outgrowth at all (Fig. 7 C). These results further support the notion that activation of $S1P_1$ by S1P may be required for NGF-induced neurite extension.

SphK1 plays an important role in neurite extension

As SphK1 activity was required for NGF-induced neurite extension in PC12 cells, it was of interest to determine whether it also played such a role in primary DRG neurons. We used D,L-*threo*-dihydrosphingosine and *N*,*N*-dimethylsphingosine (DMS), competitive inhibitors of SphK1 that block formation of S1P in PC12 cells (Edsall et al., 1997). Both inhibited neurite extension of DRG neurons in a dose-dependent manner (Fig. 8 A). DMS was somewhat more potent and was thus used in further experiments to define the importance of SphK1 in DRG neurite formation. If the effect of DMS is mediated specifically by inhibiting production of S1P, which in turn is required for stimulation of $S1P_1$, then exogenous S1P should bypass this block and reverse the effects of DMS. Importantly, suppression of neurite extension by DMS was completely rescued by exogenous S1P (Fig. 8, A and B), suggesting that the S1P produced by SphK1 is essential for neurite extension in these neurons. Moreover, pretreatment with PTX to block $S1P_1$ G_i-coupled downstream signaling (Fig. 8 A) completely prevented the ability of exogenous S1P to reverse the inhibition of neurite extension induced by DMS, pointing to an important role of $S1P_1$ activation as a consequence of activation of SphK1 and formation of S1P.

To further substantiate the role of SphK1, we also used small interfering RNA (siRNA) targeted to SphK1. In agreement with the SphK inhibitor results (Fig. 8 A), transfection of dissociated DRG neurons with siRNA targeted to SphK1 decreased neurite extension by nearly 50%, whereas control siRNA had no significant effects (Fig. 8 C). This reduction

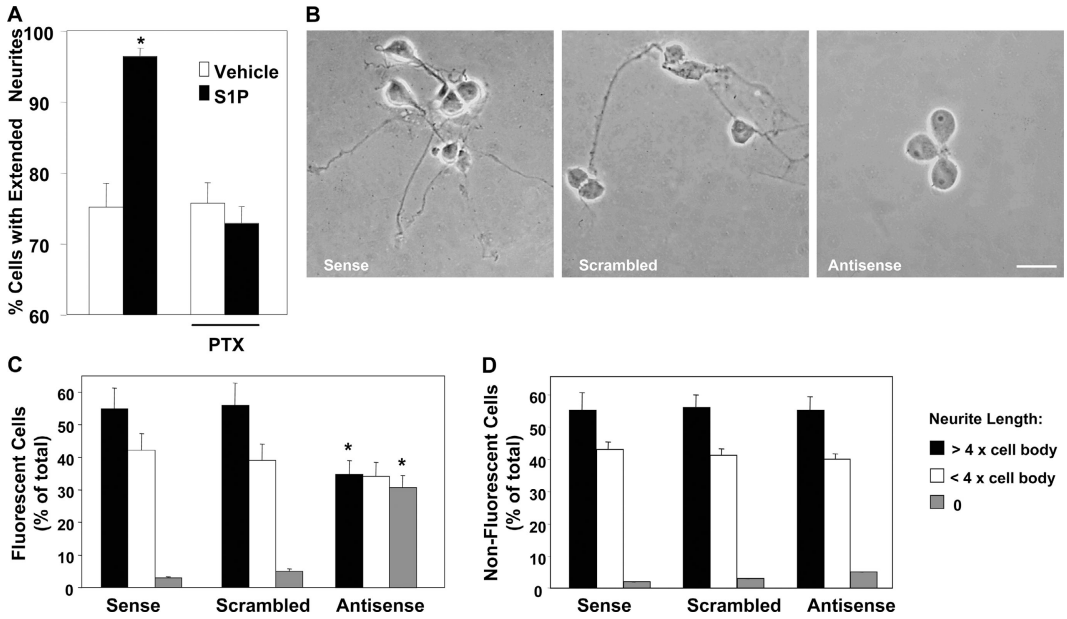

Figure 7. **$S1P_1$ mediates neurite extension.** (A) Rat E15 DRG neurons were dissociated, plated on growth factor–reduced Matrigel™-coated coverslips, and exposed to vehicle (open bars) or 100 nM S1P (filled bars) for the final 90 min of a 16-h post-dissection culture period in 100 ng/ml NGF-containing medium. Where indicated, 200 ng/ml PTX was added during the last 3 h. Neurite extension was quantified at 16 h by assessing the percentage of cells bearing at least one neurite four times the length of the cell body. Asterisks in A and C denote significant differences relative to untreated cells ($P < 0.01$, ANOVA, Tukey's). (B and C) Rat E15 DRG neurons were dissociated and transfected with $S1P_1$ antisense, sense, or scrambled fluorescent oligonucleotides for 120 h. (B) Photomicrographs of representative neurons. Bar, 50 μm. (C) Neurite extension was quantified by assessing the percentage of fluorescent neurons bearing at least one neurite four times the length of the cell body (black bars), the percentage of neurons bearing neurites less than four times the length of the cell body (white bars), and the percentage of neurons bearing no neurites (gray bars). (D) Nonfluorescent (nontransfected) neurons in the same cultures were also quantified similarly. A minimum of 100 neurons was examined and bars represent the averages ± SD. Similar results were obtained in two independent experiments.

correlated with the down-regulation of SphK1 mRNA as determined by RT-PCR (Fig. 8 D).

Discussion

The importance of the local signals that promote axonal growth of peripheral neurons, particularly the ERK and PI3K pathways, coordinated by TrkA in response to NGF, are now beginning to be elucidated (Kaplan and Miller, 2000; Markus et al., 2002a). In cultures of sympathetic neurons, it was shown that the PI3K pathway is required for NGF-induced distal axon growth (Ming et al., 1999) and plays an important role in neuritogenesis of adult rat DRG sensory neurons (Kimpinski and Mearow, 2001). PI3K may regulate axonal growth cone actin cytoskeleton through its effectors Rac and Akt (Markus et al., 2002a). Although the involvement of ERK in NGF-induced neurite outgrowth in PC12 cells is well established (Vaudry et al., 2002), its role in nervous system development is much more complex. NGF-stimulated ERK is not necessary for neurite outgrowth of chick DRG neurons (Klinz et al., 1996) or mouse DRG neurons (Klesse and Parada, 1998). However, a recent report (Markus et al., 2002b) with sensory neurons lacking the proapoptotic protein Bax enabled the examination of individual signaling mediators on axonal growth without affecting survival. Although Ras was both necessary and sufficient for NGF-stimulated axon growth, the Ras effector Raf, an activator of the ERK pathway, and Akt, known to be activated by PI3K, mediated distinct morphological features of developing neurons (Markus et al., 2002b). TrkB, which stimulates signaling proteins in a manner similar to TrkA, mediates both neuronal survival and axonal outgrowth in rat sympathetic neurons by activating the PI3K and ERK signaling pathways (Atwal et al., 2000). Inhibitors of MEK–ERK and PI3K blocked axon growth from naive and NGF-stimulated E13 sensory neurons without effecting elongation of adult sensory neurons after a conditioning lesion, suggesting that the signaling mediators that underlie regenerative axon growth are distinct from those used during development (Liu and Snider, 2001).

In this report, we describe an extended paradigm for TrkA signaling in regulation of neurite extension. According to this model, activation of TrkA by NGF stimulates and translocates SphK1 from the cytosol to the plasma membrane, where its substrate sphingosine resides, to generate S1P in a spatially and temporally restricted manner. This S1P binds to and activates $S1P_1$ on the cell surface, leading to activation of signaling pathways important for neurite extension. Our results also suggest that differential cross-communication of TrkA with S1P receptors, a family of GPCRs that regulates opposing downstream signals important for cytoskeletal changes, modulates neurite extension or retraction.

In support of this notion, it was recently shown that TrkA is tethered to the classical GPCR signaling molecules, GPCR kinase 2 and β-arrestin, providing a platform for integrative signaling by these two receptors to promote differentiation in PC12 cells (Rakhit et al., 2001). In addition, GAIP, a regulator of G protein–signaling protein, forms a

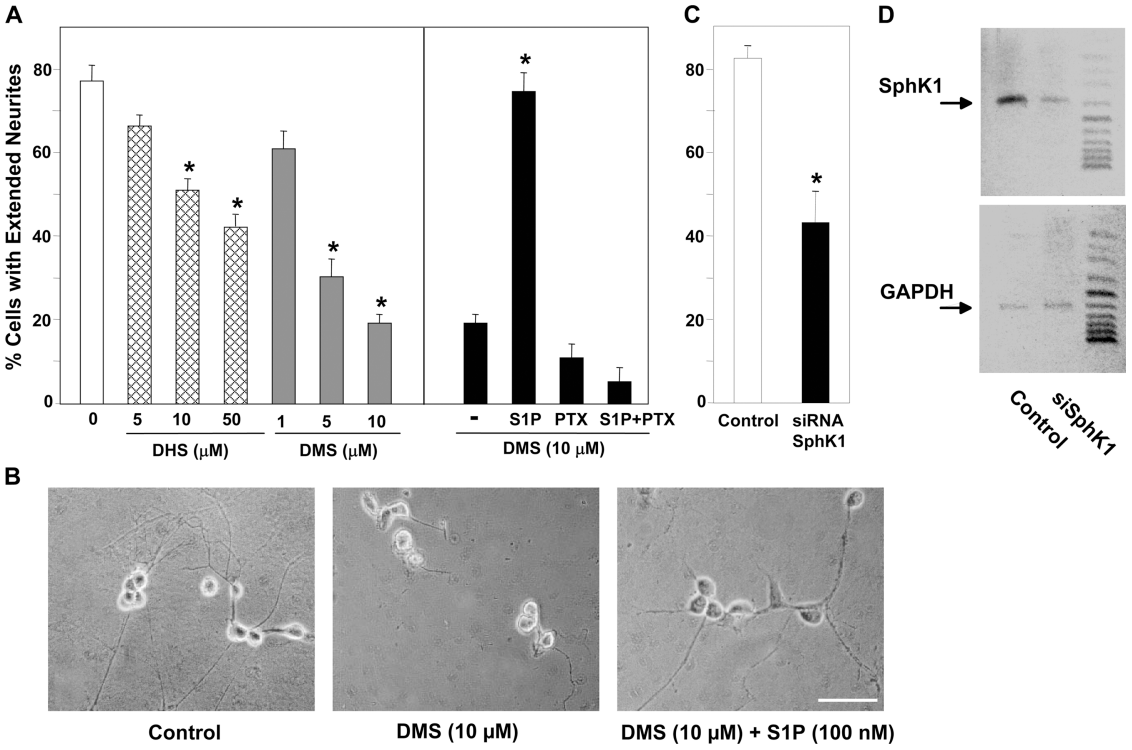

Figure 8. **SphK1 activity is required for neurite extension.** (A) Rat E15 DRG neurons were dissociated, plated on growth factor–reduced Matrigel™-coated coverslips, and treated with the indicated concentrations of D,L-*erythro*-dihydrosphingosine (DHS, hatched bars) or *N*,*N*-dimethylsphingosine (DMS, gray bars), and were cultured for 16 h in the presence of NGF. Parallel cultures (right) incubated with 10 μM DMS (black bars) were subsequently treated with 200 ng/ml PTX for the final 3 h, 100 nM S1P for the final 1.5 h, or both. Neurite extension was quantified. (B) Photomicrographs of representative neurons examined by phase microscopy are shown. Note that S1P rescued the DMS-inhibited neurite extension. Bar, 100 μm. (C) Rat E15 DRG neurons were dissociated and transfected with a control siRNA sequence or with siRNA targeted to SphK1. After 120 h in the presence of NGF, neurite outgrowth was quantified by assessing the percentage of neurons bearing neurites four times the length of their cell bodies. Asterisks in A and C denote significant differences relative to untreated cells ($P < 0.01$, ANOVA, Tukey's). (D) RT-PCR analysis of SphK1 and GAPDH expression in DRGs transfected with control siRNA or siSphK1.

complex with TrkA and is a putative link between G protein and receptor tyrosine kinase pathways (Lou et al., 2001).

Previously, we suggested that PDGF-induced cell migration required $S1P_1$ (Hobson et al., 2001; Rosenfeldt et al., 2001), representing a similar mechanism of crosstalk between receptor tyrosine kinases and GPCRs. However, others have questioned the generality of the concept that SphK1 and $S1P_1$ are downstream elements of PDGF-induced chemotaxis (Kluk et al., 2003). Recently, several different mechanisms for $S1P_1$ and receptor tyrosine kinase cross-communication have been suggested (Lee et al., 2001; Waters et al., 2003). In the integrative signaling model, the PDGF receptor and $S1P_1$ form complexes that are cointernalized together by PDGF as a functional signaling unit to regulate ERK1/2 (Waters et al., 2003). A second proposal suggests that tyrosine kinase receptors, such as the IGF-1 receptor, could transactivate $S1P_1$ through its Akt-dependent phosphorylation in a manner that does not require the SphK pathway (Lee et al., 2001). Both of these models suggest that activation of SphK1 and intracellular generation of S1P do not play any role and introduce the concept of ligand-independent activation of S1P receptors.

In contrast, several lines of evidence support a central role for SphK1 and generation of S1P in NGF-induced neurite extension. Previously, we found that NGF rapidly stimulated SphK1 in PC12 cells that was followed by a prolonged increase in SphK activity and a concomitant increase in S1P levels (Edsall et al., 1997). The activation of SphK1 by NGF, but not by FGF, was blocked by K252a, an inhibitor of TrkA (Edsall et al., 1997). Here, we demonstrated that NGF induced translocation of SphK1 from the cytosol to the plasma membrane in PC12 cells and in primary DRG neurons. Moreover, overexpression of SphK1 in PC12 cells enhanced initiation of NGF-induced neuritogenesis. Conversely, a catalytically inactive mutant SphK1, which was also translocated by NGF to the plasma membrane, markedly reduced both initiation and extension of neurites. Interestingly, active SphK1 was required for NGF-induced activation and internalization of S1P receptors in PC12 cells, supporting the notion that formation of S1P in the vicinity of its receptors induces their activation. In agreement, it was recently reported that activation of PKC leads to translocation of SphK to the plasma membrane (Johnson et al., 2002). Importantly, SphK1 also plays a significant role in axonal growth of primary neurons. Inhibition of SphK1 in dissociated DRG neurons by pharmacological or molecular approaches drastically reduced NGF-induced neurite extension. However, most notably, addition of exogenous S1P to neurons in which SphK1 was inhibited reversed the inhibitory effects on neurite extension. Collectively, these results

suggest that activation of SphK1 and formation of S1P plays an important role during NGF-induced neurite extension of sympathetic neurons.

Neurite extension and retraction are complex processes important in formation of neural networks. Our findings suggest that transactivation of distinct S1P receptors by NGF via TrkA regulates neuronal development in a reciprocal manner whereby $S1P_1$ acts in opposition to $S1P_2$ and $S1P_5$ to coordinate neurite extension and morphological differentiation. Because the different S1P receptors are associated with a variety of opposing downstream signaling pathways, their temporally selective expression on the cell surface could consequently regulate neurite extension or retraction. In agreement, in PC12 cells, NGF-induced neuronal differentiation down-regulates expression of $S1P_2$ and $S1P_5$ (Glickman et al., 1999). Similarly, we have found that S1P has opposite effects on neuritogenesis of dissociated DRG neurons, depending on which S1P receptors are expressed. S1P inhibited neurite extension of freshly isolated DRG neurons, which express $S1P_1$ and $S1P_2$. In striking contrast, when S1P was added to DRG neurons that had already initiated neurite outgrowth and then only expressed $S1P_1$, it dramatically enhanced neurite extension.

In concordance with the opposite actions of $S1P_1$ compared with $S1P_2$ and $S1P_5$, down-regulation of $S1P_2$ by antisense oligonucleotides has been shown to enhance neurite extension and prevent S1P-induced rounding in PC12 cells (MacLennan et al., 2000). Moreover, addition of S1P to PC12 cells transiently expressing $S1P_2$ caused cell rounding (Van Brocklyn et al., 1999). In agreement, we found that overexpression of either $S1P_2$ or $S1P_5$ in PC12 cells inhibited NGF-induced neuritogenesis, whereas overexpression of $S1P_1$ enhanced it, even in the absence of exogenous S1P. Similarly, in DRG neurons, inhibition of neurite extension by S1P is primarily regulated through $S1P_2$, whereas S1P signaling through $S1P_1$ enhances neurite extension. Thus, down-regulation of $S1P_1$ expression by antisense RNA, or inactivation of its G_i-coupled signaling, completely abolished neurite extension induced by S1P. Additionally, PTX blocked the S1P "rescue" of SphK1-inhibited DRG neurons, suggesting once again that transactivation of $S1P_1$ through SphK1 activation is critical for neurite elaboration.

Remodeling of the actin cytoskeleton provides the basis for both the motility of growth cones, the highly dynamic structures at the tips of axons, and neurite extension in response to neurotrophins. Recent analyses suggest that NGF signals through TrkA to stimulate Rac1 (Nusser et al., 2002), which in turn induces transient Rho inactivation during the initial phase of neuritogenesis (Nusser et al., 2002). Conversely, RhoA, acting through Rho-associated kinase, down-regulates NGF-induced Rac1 activation to inhibit neurite formation (Yamaguchi et al., 2001). In agreement, we found that the antagonistic actions of $S1P_1$ and $S1P_2$ on neurite extension correlate with their opposing effects on Rho and Rac. Activation of $S1P_2$ elicits Rho-coupled stress fiber assembly and negatively regulates Rac activity (Okamoto et al., 2000). Although NGF activates Rac and represses Rho in $S1P_1$-overexpressing PC12 cells, it represses Rac and activates Rho in $S1P_2$-overexpressing PC12 cells. Moreover, inhibition of $S1P_1$ signaling by PTX decreased NGF-induced Rac activation. These results agree with previous reports implicating $S1P_1$ in Rac activation (Hobson et al., 2001; Lee et al., 2001). This diversity of signaling may be particularly important for remodeling neurons, as it provides a fine-tuning mechanism for the processes of neurite retraction and extension.

Materials and methods

Cell culture

PC12 cells and DRG neurons were cultured and transfected as described in the online supplemental materials (available at http://www.jcb.org/cgi/content/full/jcb.200402016/DC1).

Plasmid constructs and site-directed mutagenesis

The QuikChange® Site-Directed Mutagenesis Kit (Stratagene) was used to prepare catalytically inactive SphK1 (G82D mutation primers: forward, 5′-GTCCGGTGATGATCTGATGCATGAAGTGG-3′ and reverse 5′-CCACTTCATGCATCAGATCATCACCGGAC-3′). The mutated sequence was verified by DNA sequencing. Texas red–tagged oligonucleotides were obtained from Invitrogen (sense 5′-XATGGGGCCCACCAGCGTC-3′; antisense 5′-XGACGCTGGTGGGCCCCAT-3′; and scrambled 5′-XTGATCCTTGGCGGGGCCG-3′). siRNA for rat SphK1 (5′-CUGGCCUACCUUCCUGUAGdTT-3′, 5′-CUACAGGAAGGUAGGCCAGdTT-3′), and the control sequence (5′-UUCUCCGAACGUGUCACGUdTT-3′, 5′-ACGUGACACGUUCGGAGAAdTT-3′) were synthesized by Xeragon.

Quantification of neurite extension

PC12 cells transfected with the various GFP constructs were grown in the presence of 100 ng/ml NGF in RPMI 1640 supplemented with 2% heat-inactivated horse serum for 72 h. Cells were visualized by fluorescence microscopy (Eclipse TE300; Nikon). Images were collected with a CoolSNAP Camera (Roper Scientific) using MetaMorph® software (Universal Imaging Corp.). Extended neurites were defined as having a length equal to or greater than twice the cell body.

Dissociated DRG neurons were stained with rhodamine-conjugated phalloidin (Molecular Probes, Inc.) and neurite extension was quantified by fluorescence microscopy, assessing the percentage of neurons bearing at least one neurite four times the length of their cell bodies. Unless otherwise indicated, a minimum of 300 PC12 cells or DRG neurons was examined in a blinded manner and results are reported as averages (± SD). Similar results were obtained in at least three independent experiments.

Immunocytochemistry

PC12 cells and DRG neurons were fixed with 4% PFA/4% sucrose in PBS. Cells were permeabilized with cold acetone before immunostaining and were incubated in PBS containing 5% goat serum, 2% BSA, and 0.05% Tween 20 for 1 h to block nonspecific staining. Cultures were incubated overnight at 4°C with purified polyclonal rabbit IgG antibodies directed against unique S1P receptor COOH-terminal peptides ($S1P_1$ (X1093P), $S1P_2$ (X1091P), and $S1P_5$ (X1094P)) obtained from Exalpha and diluted 1:500 in PBS containing 1% goat serum and 0.05% Tween 20. Cells were washed with PBS and incubated for 1 h with rhodamine-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) diluted 1:150 in PBS containing 1% goat serum and 0.05% Tween 20 and then were mounted on slides using the ProLong® Antifade Kit (Molecular Probes, Inc.). Cultures were examined with a laser-scanning confocal microscope (FluoView 300; Olympus). No fluorescence was detected when the primary antibody was omitted or preincubated with the peptide antigen. Moreover, in agreement with previous characterization of S1P receptor antibodies (Jin et al., 2003), fluorescence was not observed in other cell types not expressing the corresponding S1P receptor. Conversely, fluorescence was enhanced by overexpression of the S1P receptor.

To examine expression of TrkA on DRG, fixed cells were permeabilized with cold acetone before immunostaining and incubated in PBS containing 5% goat serum, 2% BSA, and 0.05% Tween 20 for 1 h to block nonspecific staining. Cultures were incubated overnight at 4°C with TrkA-specific antibodies (sc-118; Santa Cruz Biotechnology, Inc.) diluted 1:250 in PBS containing 1% goat serum and 0.05% Tween 20. Cells were washed with PBS and incubated for 1 h with FITC-conjugated secondary antibody (1:150), rhodamine-conjugated phalloidin (1:500; Santa Cruz Biotechnology, Inc.), and Alexa Fluor® 647–conjugated cholera toxin subunit B (1:500, c-34778; Molecular Probes, Inc.) diluted in PBS containing 1%

goat serum and 0.05% Tween 20, and were then mounted on slides. Cultures were examined by confocal fluorescence microscopy (LSM model 510; Carl Zeiss MicroImaging, Inc.) with a 63× oil immersion objective. Quantitative image analysis was performed using LSM 510 image-processing software. Control immunohistochemistry experiments were performed by using normal serum or by omitting the primary antibodies.

To examine translocation of SphK1, DRG neurons were grown on coverslips in medium containing both 10% FBS and 100 ng/ml NGF for 4 h and then in their absence for 6 h. After NGF treatment in serum-free medium for the indicated times, neurons were fixed with 4% PFA in 50 mM Tris-HCl, pH 7.3, for 4 h. Coverslips were washed three times with 10 mM Tris-HCl, pH 7.3, containing 150 mM sodium chloride (TBS), blocked for 30 min with TBS containing 2% BSA, 0.05% Tween 20, and 5% normal donkey serum, and incubated overnight at 4°C with a rabbit pAb to mouse SphK1 (2 μg/ml; provided by Dr. T. Murate and Dr. Y. Banno, Gifa University School of Medicine, Gifa, Japan) in TBS containing 0.05% Tween 20 (Murate et al., 2001). After washing and 30 min incubation in blocking solution, cells were incubated for 2 h at RT with Cy2-conjugated donkey anti–rabbit secondary antibody (7.5 μg/ml; Jackson ImmunoResearch Laboratories) with TBS containing 0.05% Tween 20, and were visualized by confocal microscopy as described above.

Online supplemental material

Details about cell culture and transfection, statistical analysis, and activation of Rho and Rac (provided by Dr. J. Silvio Gutkind, NIH, Bethesda, MD) can be found as supplemental material. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200402016/DC1.

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