Sphingosine-1-phosphate–induced smooth muscle cell migration involves the mammalian target of rapamycin

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Background: **Vascular smooth muscle cell (SMC) migration is an important component of the development of intimal hyperplasia. Sphingosine-1-phosphate (S-1-P) is a lipid released from activated platelets with numerous cellular effects including the stimulation of SMC migration in vitro. We examined the role of the mammalian target of rapamycin and ribosomal p70S6 kinase (p70S6K) in S-1-P–induced SMC migration.**

Methods: **Rat arterial SMCs were cultured in vitro. Linear wound and Boyden microchemotaxis assays of migration were performed in the presence of S-1-P (0.01 to 100 μmol/L) with and without rapamycin (10 nmol/L). Western blotting was performed for phosphorylated and total p70S6K, ERK1/2, and p38^{MAPK} after stimulation with S-1-P (0.1 μmol/L), with and without rapamycin pretreatment. Phosphorylation of p70S6K was also assayed after S-1-P treatment in the presence and absence of inhibitors of PI3 kinase (wortmannin, WN, and LY294002, LY), Akt (AktI), p38^{MAPK} (SB203580), and MEK1 (PD98059).**

Results: **S-1-P stimulated migration of SMCs in both linear wound and Boyden chamber assays compared to control ($P < .05$); these responses were inhibited by rapamycin to below the level of control ($P < .05$ vs S-1-P alone for both assays) in a dose-dependent manner (inhibitory concentration of 50%, 10 nmol/L). S-1-P stimulated phosphorylation of ERK1/2, p38^{MAPK}, and p70S6K, which peaked at 5 minutes for ERK1/2 and p38^{MAPK} and10 minutes for p70S6K (2-fold increase over control for each, $P < .05$). Rapamycin prevented the phosphorylation of p70S6K at the Thr 389 site (which correlates with enzyme activity), reduced ERK1/2 phosphorylation, but had no effect on the Thr 421/Ser 424 site or on p38^{MAPK} phosphorylation. Wortmannin and LY294002 inhibited phosphorylation of the Thr 389 site of p70S6K. AktI and SB203580 had no effect on p70S6K, whereas PD98059 had a marginal effect.**

Conclusions: **S-1-P–induced SMC migration was completely inhibited by rapamycin, indicating that the p70S6K pathway is involved. This mechanism likely involves modulation of the ERK1/2 pathway. S-1-P stimulates phosphorylation of p70S6K in a MEK1-dependent, PI3 kinase–dependent, but Akt-independent manner. (J Vasc Surg 2005;41:91-8.)**

Clinical Relevance: **S-1-P is released from activated platelets at sites of vessel injury and contributes to the development of intimal hyperplasia after bypass grafting, angioplasty, and stenting. S-1-P is a potent pro-migratory molecule for SMCs. Rapamycin is a commonly used immunosuppressive agent that has most recently been incorporated as the biologic agent in drug eluting stents with good success in the coronary circulation. Rapamycin inhibits the mammalian target of rapamycin, which, in turn, controls the translational mechanisms of the cell. The role of translational control during S-1-P–induced SMC migration is poorly understood. This study identifies a link between the mammalian target of rapamycin translational pathway and S-1-P and demonstrates how rapamycin might interfere with another facet of a vessel's response to injury after a vascular intervention, namely by interfering with the cell signaling of factors released from platelets deposited at the injury site.**

Sphingosine-1-phosphate (S-1-P) is a bioactive sphingolipid found in human plasma and serum[1] and is released in large amounts from activated platelets (reaching micromolar concentrations in serum).[2] As a factor released from activated platelets at sites of vessel injury, S-1-P might regulate vascular smooth muscle cell (SMC) migration after endothelial injury. Previous data from our group suggest that S-1-P is a moderate chemoattractant for SMCs expressing S-1-P receptors (types 1, 2, and 3)[3,4]; others have shown that S-1-P type 1 receptor (S-I-P$_1$) is necessary for S-1-P to stimulate SMC migration.[5,6] We have also demonstrated that migration stimulated by S-1-P in vascular SMCs is G-protein dependent and involves PI3 kinase (PI3-K), ERK1/2, and p38^{MAPK}.[3,4] ERK1/2 and p38^{MAPK} control transcription in the cell. The role of translational control during migration is poorly understood. Rapamycin is a commonly used immunosuppressive agent that has most recently been incorporated as the

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biologic agent in drug eluting stents with good success in the coronary circulation. Rapamycin inhibits the mammalian target of rapamycin (mTOR), which in turn controls the kinase p70S6 (p70S6K) and translational mechanisms of the cell.[7] This report seeks to determine the role of mTOR in migration of rat aortic SMCs that naturally express a considerable amount of $S1P_1$ and to determine the upstream regulation of p70S6K after S-1-P stimulation.

METHODS

Materials. D-erythro-sphingosine-1-phosphate was purchased from Avanti Polar-Lipids (Alabaster, Ala). Polyclonal antibodies to phospho-p70S6K (both threonine 421/serine 424 and threonine 389 sites) and total p70S6K were purchased from Cell Signaling Technology (Beverly, Mass). Phospho-ERK1/2 antibody was purchased from Promega, Inc (Madison, Wis). Total ERK1/2 antibody was purchased from BD Transduction Laboratories (Lexington, Ky). Peroxidase-conjugated anti-rabbit immunoglobulin G antibody (raised in goat) was purchased from Jackson ImmunoResearch Laboratories, Inc (West Grove, Pa). Peroxidase-conjugated anti-mouse immunoglobulin G antibody (raised in goat) was purchased from Biorad Laboratories (Hercules, Calif). Rapamycin, Akt inhibitors (AktI-1, AktI-2, and AktI-3) and PD98059 were purchased from Calbiochem (La Jolla, Calif). Wortmannin, SB203580, and pertussis toxin were purchased from Sigma Chemical Co (St Louis, Mo). LY294002 was purchased from Biomol (Plymouth Meeting, Pa).

Cell culture. Primary rat aortic thoracic SMCs (male Sprague-Dawley, VSMC) in passages 10 to 20 were used. Cultured cells were kept in 100-mm^2 dishes in 6 mL Dulbecco modified Eagle medium (DMEM)/10% fetal bovine serum (FBS) with penicillin, streptomycin, and amphotericin B. Cells were maintained at 37°C in 5% CO_2, passaged before confluence, and fed twice weekly.

Wound assay. A linear wound assay was performed as described previously.[3] Briefly, rat VSMCs were grown to confluence in 60-mm^2 dishes and then starved for 24 hours, after which each dish was divided into a 2×3 grid. A linear wound was made in each hemisphere of the dish by using a pipette tip. Immediately after wounding, medium was changed to fresh DMEM (for all reagent dishes and as negative control) or 10% FBS (positive control). After the determination of a dose-response curve ($n = 3$ for control and each concentration of S-1-P), 0.1 μmol/L S-1-P (25 mg suspended in 5 mL methanol) was added to each dish for all subsequent trials. Dishes were treated with S-1-P either alone or with rapamycin (1 to 20 nmol/L). Trials were performed with methanol (15 μL) and dimethyl sulfoxide (0.1%) as controls for S-1-P and rapamycin, respectively. S-1-P did not induce DNA synthesis (as measured by ^{3}H-thymidine incorporation) or cell proliferation (as measured by manual cell counting during a 7-day period) in these cells (data not shown). Under a ×40 lens with an attached SPOT camera (Diagnostic Instruments, Inc, Sterling Heights, Mich), images were taken of the intersections of the linear wound and each grid line, resulting in eight fields per dish. Cells were allowed to migrate during a period of 24 hours at 37°C. Each field was photographed at time zero and after 24 hours. The area of each field was measured by using SPOT Advanced software, and eight fields from each dish were averaged. Trials with each reagent or inhibitor were performed in at least six separate dishes, and the results were averaged.

Boyden chamber assay. Chemotaxis was measured by using a 48-well Boyden chamber (Neuroprobe Inc, Gaithersburg, Md) and polycarbonate filters (10-μm pore size, 25 × 80 mm, polyvinyl propylene free; Neuroprobe, Inc). The filter was placed in sterile Dulbecco's Phosphate-Buffered Saline (dPBS) for 30 minutes before use. After the determination of a dose-response curve for S-1-P, all subsequent trials used 0.1 μmol/L S-1-P in PBS in the lower wells, with serum-free DMEM as negative and 10% FBS or platelet-derived growth factor (PDGF) (10 ng /mL) as positive controls. VSMCs at 60% to 70% confluence were starved overnight in serum-free DMEM, trypsinized, washed twice with dPBS, and resuspended in serum-free DMEM at a concentration of 2×10^4 cells in 50 μL. S-1-P or control reagents were added to the lower wells, and the polycarbonate membrane was placed over those wells. The cell suspension was added to the upper wells in 50-μL aliquots. For trials with rapamycin (10 nmol/L), the inhibitor was added to 2 mL of the cell suspension 1 hour before addition of cells to the upper wells. The chamber was incubated at 37°C for 5 hours, and the membrane was then removed. The cells on the upper side were scraped off, the membrane was stained with Diff Quik (VWR Scientific Products, Westchester, Pa), and the cells on the lower side of the membrane were manually counted. Four high-power fields (×400) were counted per well and averaged. Trials included eight or 12 wells per reagent or inhibitor per trial and were repeated no fewer than three times.

Western blotting for p70S6K. VSMCs were grown to 60% to 70% confluence in 100-mm^2 plates and starved for 24 hours in serum-free DMEM. Cells were stimulated with 0.1 μmol/L S-1-P alone or pretreated with inhibitors of mTOR (rapamycin, 10 nmol/L), G_i (pertussis toxin, 100 ng/mL), PI3-K (wortmannin, 1 μmol/L, and LY294002, 10 μmol/L), MEK1 (PD98059, 10 μmol/L), or $p38^{MAPK}$ (SB203580, 10 μmol/L) for 1 hour before stimulation with S-1-P. The doses and specificities of pertussis toxin, wortmannin, LY294002, PD98059, and SB203580 have been detailed previously.[3,4] Briefly, doses were chosen at approximately the inhibitory concentration of 50% for each inhibitor and have been used previously at these doses. After stimulation (see Results for time points), cells were washed with ice-cold PBS, harvested in ice-cold mitogen-activated protein kinase (MAPK) lysis buffer (50 mmol/L HEPES, 600 mmol/L NaCl, 3 mmol/L $MgCl_2$, 400 mmol/L ethylenediamine tetraacetic acid, 0.2% sodium dodecylsulfate, 1% Na deoxycholate, β-glycerophosphate, 2% Triton X-100, 1 mmol/L dithiothreitol, 0.2 mmol/L sodium vanadate, 4 μg/mL leupeptin, 0.2 mol/L phenylmethylsulfonyl fluoride, and 4 μg/mL aprotinin, at pH 7.5), and sonicated. Samples were

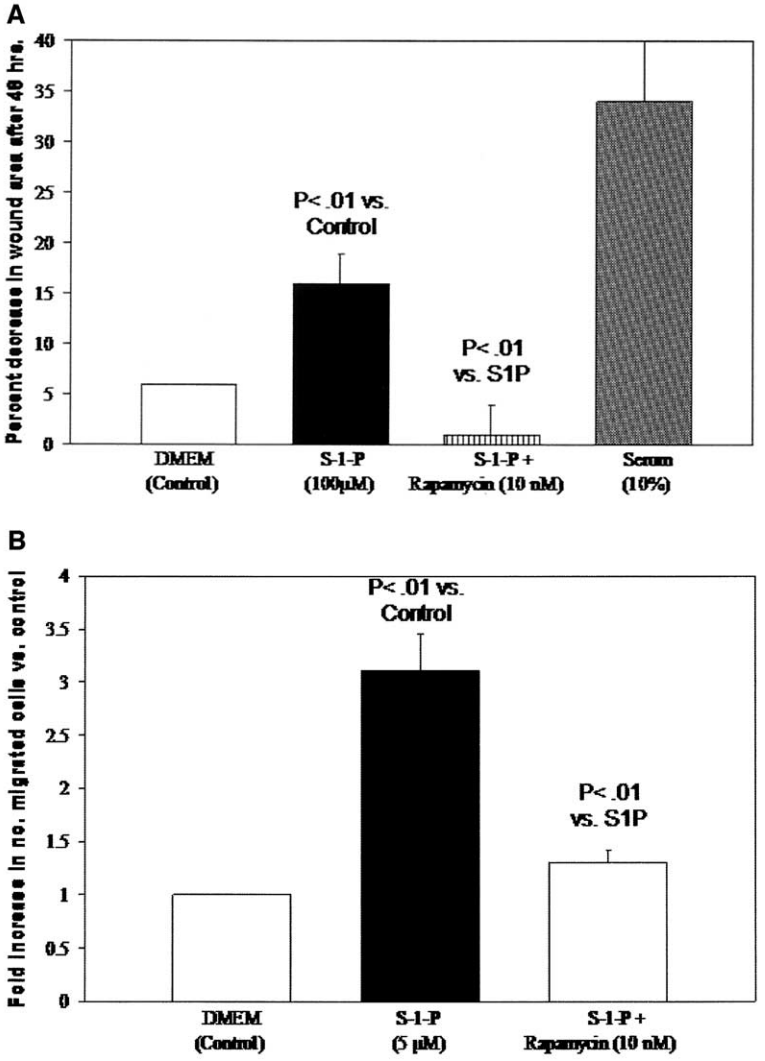

Fig 1. A, Effect of rapamycin on S-1-P–induced migration in a wound assay. Cells were allowed to migrate during 24 h in the presence of DMEM, S-1-P alone, or with rapamycin (1-10 nmol/L), or 10% FBS. A larger decrease in wound area represents greater migration across an uncoated culture dish. Values represent mean ± sem of percent decrease in wound area over six dishes (48 high-power fields [HPF]) per reagent. **B,** Effect of rapamycin on S-1-P–induced migration in a Boyden chamber assay. SMCs were washed and resuspended in DMEM at a concentration of 2×10^4 cells in 50 μL per well and then allowed to migrate through an uncoated polycarbonate membrane during 4 h. Reagents in the bottom wells were DMEM, S-1-P (5 μmol/L), or 10% FBS. Cell suspensions were pretreated for 1 h with rapamycin (10 nmol/L), an inhibitor of mTOR, in the S-1-P + Rapamycin group. Cells were counted manually, and 4 HPF per well were averaged. Values represent mean ± sem of fold increase over control (DMEM only) of at least three experiments of eight wells per reagent. Absolute cell numbers averaged 38 cells per HPF for S-1-P alone. 10% serum resulted in two-fold the migration of S-1-P alone (six-fold from control data not shown).

subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis (25 μg of protein per well) and transferred to nitrocellulose membranes. Blots were blocked for 1 hour in Tris-buffered saline with 0.1% Tween 20 and 5% non-fat dry milk for all antibodies. Blots were probed with antibodies to phospho-p70S6K at Thr 421/Ser 424 (1:1000), phospho-p70S6K at Thr 389 (1:1000), p70S6K (1:1000), phospho-ERK1/2 (1:5000), or pan-ERK1/2 (1:5000) at 4°C overnight. Blots were probed secondarily with peroxidase-conjugated anti-rabbit immunoglobulin G antibody for phospho-ERK1/2, phospho-p70S6K (both sites), and total p70S6K (Jackson, 1:10,000), or with peroxidase-conjugated anti-mouse immunoglobulin G antibody for total ERK1/2 (Biorad, 1:5000). Blots were then visualized on x-ray film by using the Enhanced Chemo-Luminescence (ECL) Western blotting detection system (Amersham Pharmacia Biotech, Arlington Heights, Ill) for peroxidase activity. Band intensity was measured by using

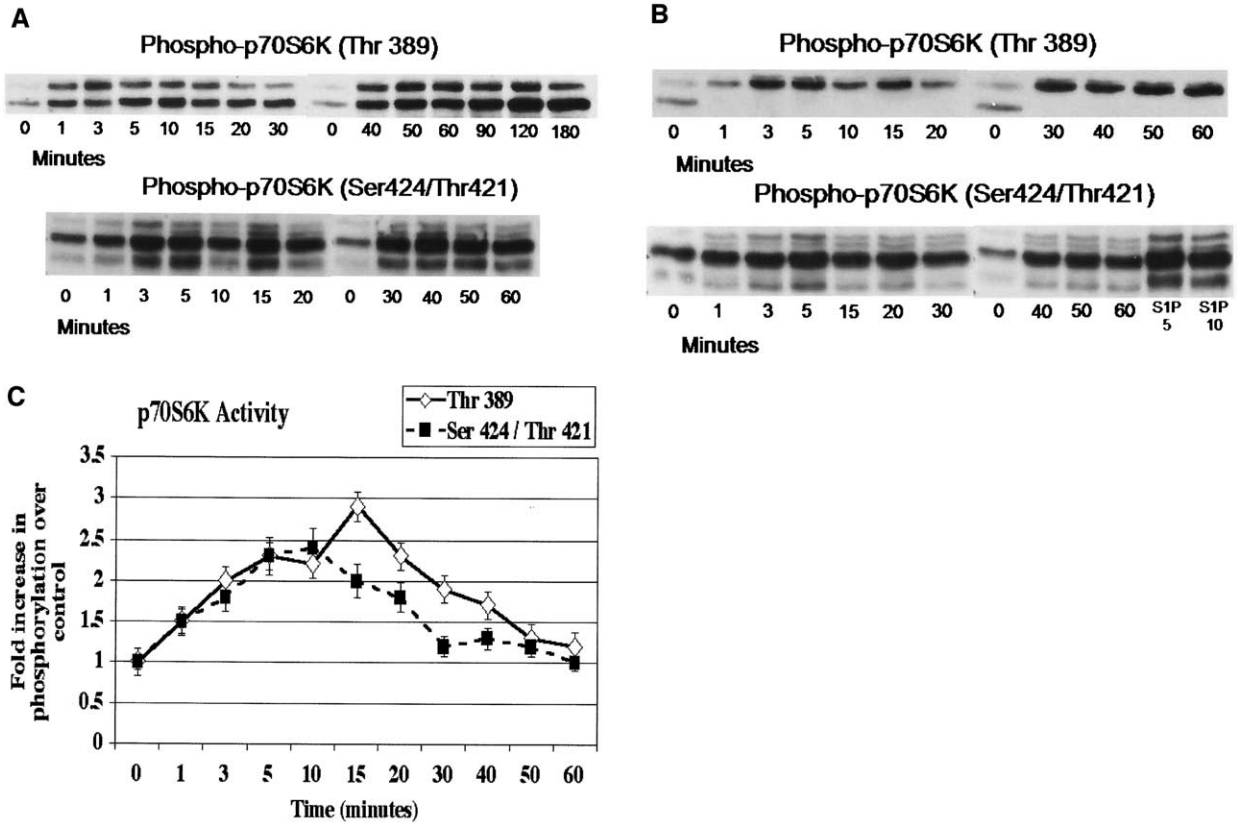

Fig 2. A, Western blots of time courses of phosphorylation of S6 kinase 1 after treatment with S-1-P (5 μmol/L). The *upper time course* represents phosphorylation at the Thr 389 site, whereas the *lower time course* represents phosphorylation at the Thr 421/Ser 424 site. The *upper band* in the Thr 389 time course represents p85S6K phosphorylation, whereas the *lower band* represents p70S6K phosphorylation. Blots are representative of three separate experiments. Time 0 is an unstimulated control. Total p70S6K did not change at any time point, and protein loading was equal for all lanes (data not shown). **B,** Western blots of time courses of phosphorylation of S6 kinase 1 by S-1-P (5 μmol/L) after 1-h pretreatment with rapamycin (10 nmol/L), an inhibitor of mTOR. There is complete dephosphorylation of the p70S6K Thr 389 site (*lower band of upper time course*). Blots are representative of three separate experiments. Time 0 is an unstimulated control without rapamycin pretreatment. *S1P 5* and *S1P 10* are positive controls (S-1-P alone) at 5 and 10 min. Total p70S6K did not change at any time point, and protein loading was equal for all lanes (data not shown). **C,** Time course of the phosphorylation of p70S6K Thr 389 site and p70S6K Thr 421/Ser 424 site during the first 60 minutes in response to S-1-P (5 μmol/L). Values represent the mean ± sem fold increase of phosphorylation over control (n = 3).

Gelimaging software (Kodak, Rochester, NY). All experiments were performed at least three times.

Statistical analysis. All data are presented as the mean ± standard error of the mean (sem). Statistical differences between groups were analyzed by using one-way analysis of variance with post hoc Dunnett's multiple comparisons correction where appropriate. A P value of $<.05$ was considered significant.

RESULTS

Rapamycin inhibits S-1-P–stimulated SMC migration. S-1-P stimulated migration of VSMCs in both the linear wound and Boyden microchemotaxis chamber assays two to three times the level of control ($P < .01$ vs control for both, Fig 1, *A* and *B*). The doses of S-1-P used for each assay were determined from previously published dose-response curves.[3,4] Rapamycin produced a concentration-dependent decrease in cell migration with an inhibitory concentration of 50% of 10 nmol/L (data not shown). The presence of rapamycin inhibited migration in both assays to the level of control ($P < .01$ vs S-1-P alone and $P =$ not significant vs control for both methods, Fig 1, *A* and *B*). Serum-stimulated migration was approximately twice the level of S-1-P for both assays (Fig 1, *A* and data not shown).

S-1-P stimulates mTOR-dependent p70S6K phosphorylation. Phosphorylation of p70S6K was used as a downstream readout of mTOR activity. p70S6K is phosphorylated at multiple sites after growth factor or amino acid stimulation[8] and requires mTOR activity for full activation. In addition, S6 kinase I exists as both p70 and p85

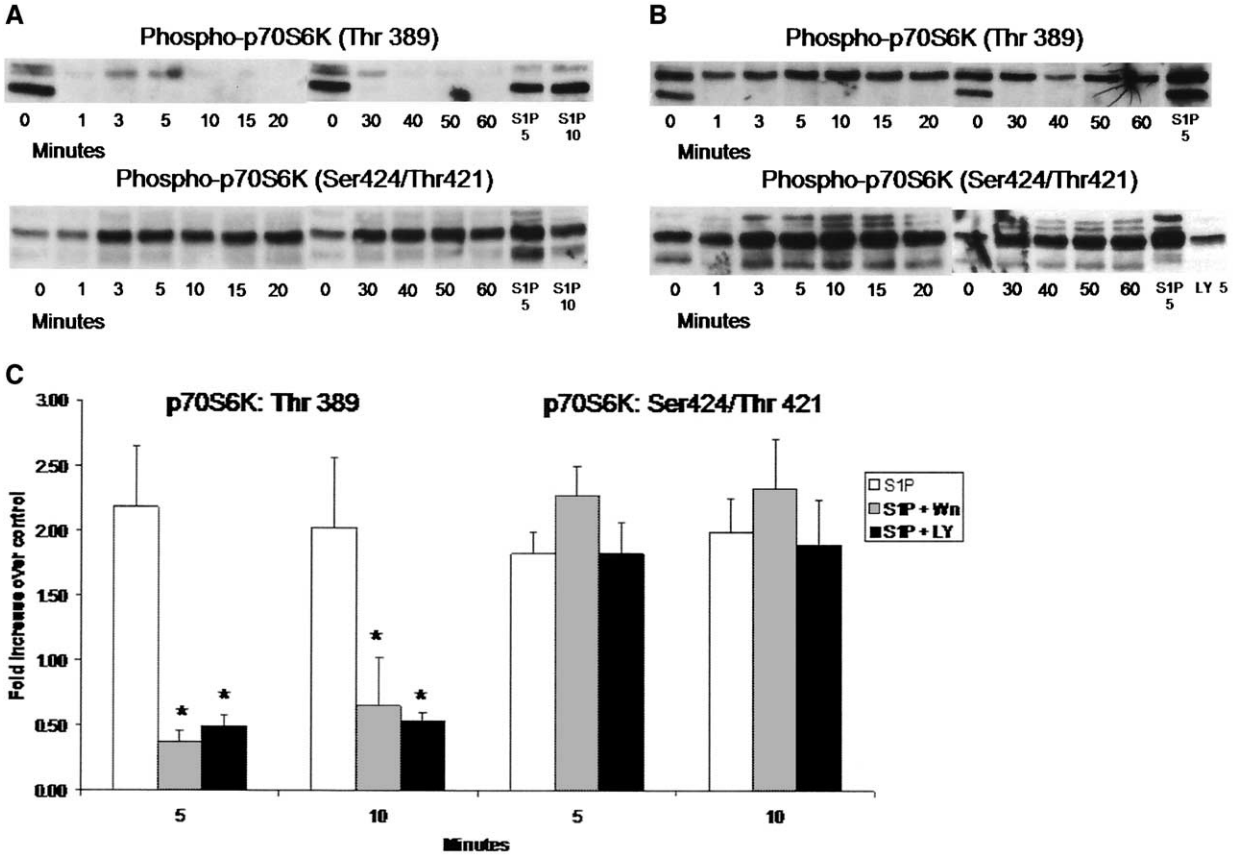

Fig 3. A, Western blots of time courses of S6 kinase 1 phosphorylation by S-1-P (5 μmol/L) after 1-h pretreatment with wortmannin (1 μmol/L), an inhibitor of PI3-K. There is complete dephosphorylation of the p70S6K Thr 389 site (*lower band of upper time course*). Total p70S6K did not change at any time point, and protein loading was equal for all lanes (data not shown). Blots are representative of three separate experiments. Time 0 is an unstimulated control without rapamycin pretreatment. *S1P 5* and *S1P 10* are positive controls (S-1-P alone) at 5 and 10 min. **B,** Western blots of time courses of S6 kinase 1 phosphorylation by S-1-P (5 μmol/L) after 1-h pretreatment with LY294002 (10 μmol/L), an inhibitor of PI3-K. There is complete dephosphorylation of the p70S6K Thr 389 site *(lower band of upper time course)*. Total p70S6K did not change at any time point, and protein loading was equal for all lanes (data not shown). Blots are representative of three separate experiments. Time 0 is an unstimulated control without rapamycin pretreatment. *S1P 5* and *S1P 10* are positive controls (S-1-P alone) at 5 and 10 min. *LY 5* is a negative control (LY294002 alone at 5 min). **C,** Phosphorylation of p70S6K at Thr 389 (5 and 10 min, *left*) and Thr 421/Ser 424 (5 and 10 min, *right*) sites with S-1-P alone (5 μmol/L) or after 1-h pretreatment with wortmannin (1 μmol/L) or LY294002 (10 μmol/L). Total p70S6K did not change at any time point, and protein loading was equal for all lanes (data not shown). Values represent mean ± sem of fold increase over control and represent the results of three separate experiments. $*P < .01$ vs S-1-P alone.

proteins; p70 is located primarily in the cytoplasm, whereas p85 is exclusively nuclear.[8] The function of the p85 form is less clear, and it seems more likely that the p70 form is involved in the migration response. Two phosphorylation sites were selected: Thr 389, a site in the linker domain, and Thr 421/Ser 424, a site in the carboxy-terminal auto-inhibitory domain. The phosphorylation state of the Thr 389 site has been found to correlate with p70S6K enzyme activity, and this site is the most rapamycin-sensitive. The Thr 421/Ser 424 site might be growth factor– or MAPK-regulated, and its phosphorylation is necessary for relief of auto-inhibition and exposure of other catalytic sites. S-1-P stimulated phosphorylation of both sites in a time-dependent manner (Fig 2, *A* and *C*). Rapamycin pretreatment for 1 hour led to complete dephosphorylation of p70S6K at the Thr 389 site, even after stimulation with S-1-P, but it had no effect on the analogous site for p85 or the Thr 421/Ser 424 site (Fig 2, *B*). These data suggest that mTOR activity is necessary for p70S6K phosphorylation at the Thr 389 site, and that this regulation is specific to p70S6K after 1 hour pretreatment with rapamycin.

PI3-K regulates p70S6K phosphorylation by S-1-P. We have demonstrated previously that inhibition of PI3-K with wortmannin inhibits S-1-P–induced SMC mi-

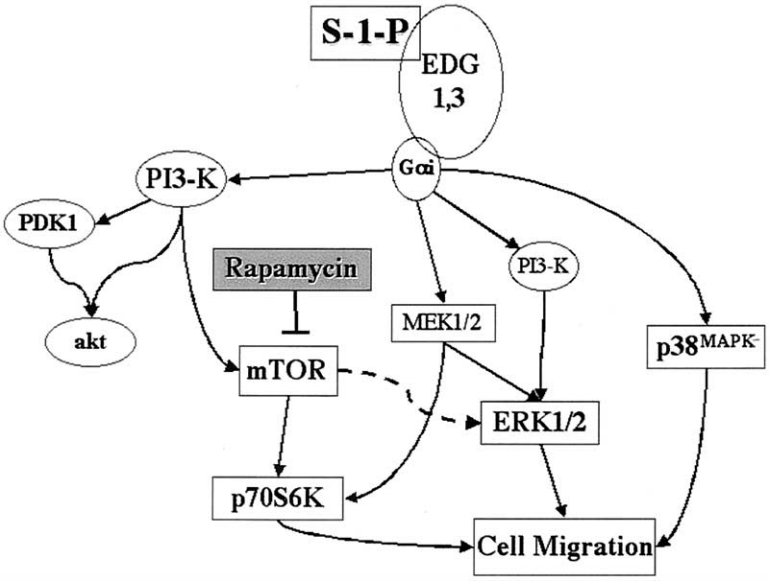

Fig 4. S-1-P and mTOR: S-1-P activates the *EDG-1* and *-3* receptors on SMCs, leading to activation of a Gαi protein. MAPK pathways ERK1/2 and $p38^{MAPK}$ are activated and lead to cell migration. PI3-K is also activated, leading to phosphorylation of ERK1/2, PDK1, and Akt. mTOR is upstream of P70S6K and can be inhibited by rapamycin, leading to a decrease in p70S6K activation. This report suggests that p70S6K is downstream of PI3-K and MEK1/2 and that there appears to be Akt-independent activation of p70S6K. There is crosstalk with mTOR and the ERK1/2 pathway. Inhibition of mTOR by rapamycin results in the inhibition of cell migration in response to S-1-P.

gration[3] and that S-1-P stimulates PI3-K–dependent Akt phosphorylation.[4] Similar to rapamycin, pretreatment with wortmannin and LY294002 resulted in complete dephosphorylation of p70S6K, even after S-1-P addition (Fig 3, *A-C*). There was no effect on p85 phosphorylation or on the Thr 421/Ser 424 site. These results suggest that PI3-K activity is necessary for p70S6K phosphorylation at the Thr 389 site, and that this regulation is specific to p70S6K (as with rapamycin). Akt is one of the downstream kinases linked to PI3-K and is considered to modulate S6K in some cells. Incubation with Akt inhibitors blocked Akt phosphorylation (data not shown) but had no effect on S6K phosphorylation at the Thr 389 site. These data support a role for Akt independent activation of mTOR/p70S6K in response to S-1-P (see schema Fig 4).

MEK1/2 but not $p38^{MAPK}$ might regulate p70S6K phosphorylation by S-1-P. We have previously shown that S-1-P stimulates SMC migration that is inhibited by PD98059 and SB203580 (inhibitors of MEK1 and activated $p38^{MAPK}$, respectively).[3,4] Pretreatment with rapamycin does affect ERK1/2 phosphorylation at 10, 15, and 20 minutes (Fig 5, *A*) but does not affect $p38^{MAPK}$ phosphorylation by S-1-P at any time point (data not shown). This must suggest that the effects of rapamycin on migration might in part be due to inhibition of ERK1/2 kinases. SB203580 pretreatment had no effect on S-1-P–induced phosphorylation of either phosphorylation site on p70S6K (Fig 5, *B* and *D*). Pretreatment with PD98059 did reduce phosphorylation of p70S6K at the Thr 389 and Thr 421/Ser 424 sites (Fig 5, *C* and *D*), suggesting that MEK1 does regulate p70S6K phosphorylation (see schema Fig 4).

DISCUSSION

Cell migration is a complex program that allows a cell to move through tissue and is a principal process in wound healing. Both transcription and translation are required to provide the proteins necessary to achieve migration. Control of translation is mediated by the influence of mTOR on the activity of p70S6K, which regulates many key cell cycle and nuclear events involved in cell growth and function.[9-11] mTOR is regulated by the TSC1/TSC2 complex[12] and can be inhibited by rapamycin. Rapamycin has been shown to block both SMC proliferation and migration in response to serum, basic fibroblast growth factor (bFGF),[13] and PDGF.[14] The association of mTOR/p70S6K with the cytoskeleton is tentative, but it does appear that rapamycin can also prevent Ca^{2+} associated stress fiber formation,[15] and that p70S6K can be co-localized with actin stress fibers near the forward edge of migrating cells.[16] Insertion of hamartin, a protein that inhibits mTOR activity, will interrupt p70S6K signaling and block cell migration.[17] Competitive displacement of rapamycin with FK406 prevents the inhibition of rapamycin on cell migration.[18] Collectively, these data suggest a role for mTOR-regulated p70S6K in cell migration.

We have demonstrated that S-1-P stimulates migration that is inhibited completely by rapamycin pretreatment in a concentration-dependent manner. With pharmacologic in-

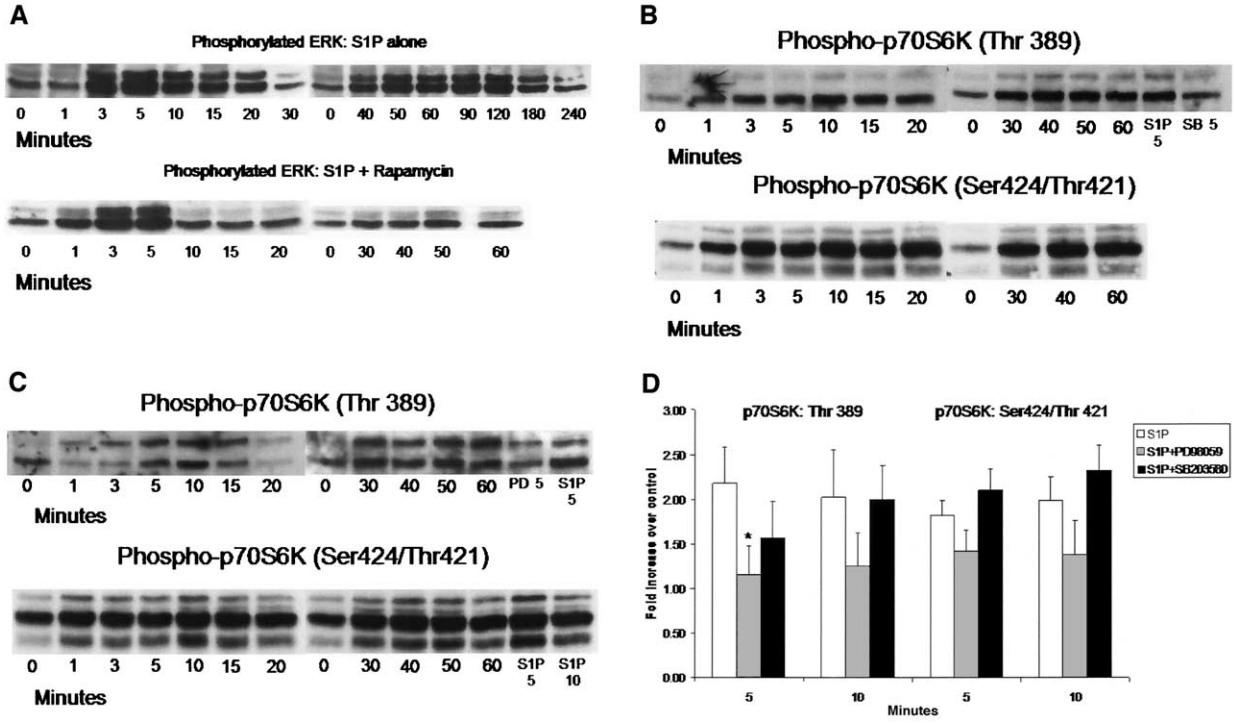

Fig 5. A, Western blots of time courses of ERK1/2 phosphorylation by S-1-P alone (5 μmol/L) or after 1-h pretreatment with rapamycin (10 nmol/L). Blots are representative of the results of three separate experiments; there were significant differences between the rapamycin-treated and untreated groups at 10, 15, and 20 minutes. There was no change in total ERK1/2 at any time point, and protein loading was equal for all lanes (data not shown). Time 0 is an unstimulated control. **B,** Western blots of time courses of S6 kinase 1 phosphorylation with S-1-P (5 μmol/L) after 1-h pretreatment with SB203580 (10 μmol/L), an inhibitor of activated $p38^{MAPK}$. Total p70S6K did not change at any time point, and protein loading was equal for all lanes (data not shown). Blots are representative of the results of three separate experiments. Time 0 is an unstimulated control without SB203580 pretreatment. *S1P 5* is S-1-P alone at 5 min (positive control), and *SB 5* is SB203580 alone (negative control) in the Thr 389 time course. **C,** Western blots of time courses of S6 kinase 1 phosphorylation with S-1-P (5 μmol/L) after 1-h pretreatment with PD98059 (10 μmol/L), an inhibitor of MEK1. Total p70S6K did not change at any time point, and protein loading was equal for all lanes (data not shown). Blots are representative of the results of three separate experiments. Time 0 is an unstimulated control without PD98059 pretreatment. *S1P 5* and *S1P 10* are S-1-P alone at 5 and 10 min (positive controls); *PD 5* is PD98059 alone (negative control) in the Thr 389 time course. **D,** Phosphorylation of p70S6K at Thr 389 (5 and 10 min, *left*) and Thr 421/Ser 424 (5 and 10 min, *right*) sites with S-1-P alone (5 μmol/L) or after 1-h pretreatment with PD98059 (10 μmol/L) or SB203580 (10 μmol/L). Values represent mean ± sem of fold increase over control and represent the results of three separate experiments. $*P = .05$ vs S-1-P alone.

hibitors, we found that S-1-P stimulates phosphorylation of p70S6K through mTOR- and PI3-K–dependent mechanisms, and that the MAP kinases ERK1/2 and $p38^{MAPK}$, which independently regulate SMC migration stimulated by S-1-P,[3,4] do not appear to regulate p70S6K phosphorylation. Whereas S-1-P stimulates phosphorylation of p70S6K at both Thr 389 and Thr 421/Ser 424 sites, only the Thr 389 site is regulated by mTOR and PI3-K. Phosphorylation of the p85 form of the protein, which is exclusively nuclear,[9] at the site corresponding to Thr 389 on p70S6K is not affected by rapamycin. In contrast to several reports, we did not find a role for Akt in the activation of p70S6K.[19] Recent data suggest that the tuberous sclerosis gene products (TSC1 and TSC2) interact in a functional complex that might represent the connection between the PI3-K/Akt/PDK and mTOR pathways in both *Drosophila* and mammalian cells.[20] Those experiments also emphasized the specific regulation of the Thr 389 site by mTOR and PI3-K pathways.

Recent reports showed inhibition of migration stimulated by bFGF in mouse aortic SMCs[13] and by PDGF-BB in rat and human SMCs[14] after 48 hours of rapamycin pretreatment. However, both studies found no effect with rapamycin pretreatment up to 6 hours. The long pretreatment in both studies might have altered the proportion of cells in G_1/S phases that might be more prone to migrate after stimulation; rapamycin might not specifically antagonize migration in response to these agonists. Differences from our findings might be due to the fact that PDGF and bFGF are stronger stimulants of migration than S-1-P in

SMCs, and the presence of multiple parallel signaling pathways might negate the effects of inhibition of mTOR alone with these agonists. In addition, S-1-P receptors initially couple exclusively to G-proteins, whereas PDGF and bFGF stimulate tyrosine kinase receptors specifically (although receptor transactivation likely occurs). Kluk and Hla[5] reported no effect of rapamycin pretreatment on migration of their rat SMCs after S-1-P stimulation, but in contrast to our SMCs,[4] their adult cells expressed very little S-1-P_1, and S-1-P only stimulated migration after transfection of S-1-P_1 in their model. Although the link between PDGF and S-1-P in migration has already been established,[6] a more recent report has suggested that S-1-P can potently stimulate PDGF-A and PDGF-B chain expression through both ERK1/2 and $p38^{MAPK}$ pathways' activation of AP-1.[21] It is possible that mTOR/p70S6K is involved in the translation of the PDGF-A and B chains, and this would contribute to long-term migration in response to S-1-P. Although such a mechanism would be involved in the wound assay in these experiments, the short duration of the Boyden chamber is likely insufficient for this mechanism to contribute to the migration response.

A novel finding is that rapamycin inhibited ERK1/2 activation. This appears not to be a nonspecific effect, because neither $p38^{MAPK}$ nor Akt was not inhibited by rapamycin. This crosstalk has been identified recently in VSMCs by others.[22] How this crosstalk between the ERK1/2 pathway and mTOR/p70S6K might occur is as yet not defined. Inhibition of $p38^{MAPK}$ with SB203580 had no significant effect on p70S6K phosphorylation at either site tested. Pretreatment with PD98059, a MEK1 inhibitor, did reduce phosphorylation of p70S6K at the Thr 389 and Thr 421/Ser 424 sites, suggesting that MEK1 might partially regulate p70S6K phosphorylation. It has been shown in cell lines that phosphorylation and inactivation of TSC2 can occur through a PI3-K–dependent but Akt-independent pathway that involves either protein kinase C– or MEK-mediated mechanisms.[23] Although some evidence suggests that the Thr 421/Ser 424 site is growth factor–regulated,[8,24] the current Western blotting data show no reduction in phosphorylation at this site with inhibition of mTOR, PI3-K, Akt, ERK1/2, or $p38^{MAPK}$ before S-1-P treatment.

In conclusion, S-1-P–mediated cell migration requires ribosomal p70S6 kinase through a PI3-K–dependent manner, Akt independent, and involves mTOR. This mechanism likely involves modulation of the ERK1/2 pathway (see schema Fig 4). S-1-P is released from activated platelets at sites of injury and is also a secondary mediator of PDGF on SMCs. The effect of rapamycin on S-1-P signaling adds another facet to mechanisms in vivo, whereby rapamycin might interfere with the response to injury after vascular intervention.

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