Ceramide 1-Phosphate Mediates Endothelial Cell Invasion via the Annexin a2-p11 Heterotetrameric Protein Complex

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The bioactive sphingolipid, ceramide 1-phosphate (C-1-P), has been implicated as an extracellular chemotactic agent directing cellular migration in hematopoietic stem/progenitor cells and macrophages. However, interacting proteins that could mediate these actions of C-1-P have, thus far, eluded identification. We have now identified and characterized interactions between ceramide 1-phosphate and the annexin a2-p11 heterotetramer constituents. This C-1-P-receptor complex is capable of facilitating cellular invasion. Herein, we demonstrate in both coronary artery macrovascular endothelial cells and retinal microvascular endothelial cells that C-1-P induces invasion through an extracellular matrix barrier. By employing surface plasmon resonance, lipid-binding ELISA, and mass spectrometry technologies, we have demonstrated that the heterotetramer constituents bind to C-1-P. Although the annexin a2-p11 heterotetramer constituents do not bind the lipid C-1-P exclusively, other structurally similar lipids, such as phosphatidylserine, sphingosine 1-phosphate, and phosphatidic acid, could not elicit the potent chemotactic stimulation observed with C-1-P. Further, we show that siRNA-mediated knockdown of either annexin a2 or p11 protein significantly inhibits C-1-P-directed invasion, indicating that the heterotetramer complex is required for C-1-P-mediated chemotaxis. These results imply that extracellular C-1-P, acting through the extracellular annexin a2-p11 heterotetrameric protein, can mediate vascular endothelial cell invasion.

Background: Extracellular ceramide 1-phosphate is presumed to interact with extracellular proteins to mediate cellular invasion. These proteins are unidentified.

Results: C-1-P interacts with both annexin a2 and p11 proteins. C-1-P-mediated vascular endothelial cell invasion requires expression of these proteins.

Conclusion: Extracellular C-1-P mediates invasion via an interaction with the annexin a2-p11 heterotetramer.

Significance: Gradients of C-1-P may guide vascular endothelial cell invasion during wound healing.

The anionic sphingolipid ceramide 1-phosphate (C-1-P), and the anabolic enzyme ceramide kinase, which generates C-1-P, enhance cellular migration and chemotaxis of circulating hematopoietic stem/progenitor cells and macrophages (1–4). C-1-P is readily secreted from cells (5) and can elicit selective actions dependent on localization within an intra- or extracellular compartment. Although there is evidence that extracellular C-1-P mediates macrophage migration through a pertussis toxin-sensitive G-protein-coupled receptor (1), the identity of an extracellular receptor for C-1-P is unknown. It is also unclear what effect circulating, extracellular C-1-P might have on the vascular endothelium.

The concept of immunomodulation by C-1-P has become increasingly nuanced. It is firmly established that C-1-P activates cytosolic phospholipase A2 through an allosteric site, leading to the production of proinflammatory arachidonic acid metabolites (6–9). However, ample evidence now indicates that C-1-P negatively regulates inflammatory cytokine production. We have shown, for example, that exogenous C-1-P abrogates LPS-mediated production of TNF-α, IL-6, IL-8, and IL-1β in human peripheral blood mononuclear cells (10). Additionally, recent evidence documents the direct inhibition of TNF-α-converting enzyme by C-1-P binding (11). These data supported previous research demonstrating that C-1-P, produced via ceramide generated from acid sphingomyelinase activity, could negatively modulate TNF-α release (12, 13).

The abbreviations used are: C-1-P, ceramide 1-phosphate; A2t, annexin a2-p11 heterotetramer; ASA, acetylsalicylic acid; cPLA2, cytosolic phospholipase A2; DOPC, phosphatidylcholine; DOPS, phosphatidylserine; HCAEC, human coronary artery endothelial cell(s); HREC, human retinal microvascular endothelial cell(s); HSPC, hematopoietic stem/progenitor cell(s); PA, phosphatidic acid; RU, response unit(s); S-1-P, sphingosine 1-phosphate; SDF-1, stromal derived factor-1; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

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The evidence indicating that C-1-P might differentially regulate autocrine and paracrine inflammatory mediators led us to this study. Our goal was to investigate the mechanisms by which extracellular C-1-P might interact with vascular endothelial cells to regulate wound healing processes. Vascular wound healing requires recruitment of circulating immune cells and spatiotemporal production of chemokines, cytokines, and coagulation/fibrinolytic factors to initiate repair (14). However, wound healing processes after vascular stenting can go awry and contribute to prolonged inflammation and neointimal hyperplasia (15). We have now examined the role of C-1-P in endothelial cell invasion through a newly characterized mechanism and comment on its potential contribution to vascular wound healing.

We began this work after conducting a screen for proteins that interacted with sucrose-loaded vesicles of C-1-P. After identifying the interacting proteins by mass spectrometry, our results suggested that the annexin a2 protein bound to C-1-P. The annexin family of proteins shares a structural motif of four annexin repeats, folded into a domain that allows transient alignment with membrane lipids (16). Annexin a2 shares common features with other annexin family members, such as binding to anionic phospholipids in a calcium-dependent manner (17, 18). However, annexin a2 is distinguished from other annexins by its regulation of (19, 20) and interaction with the annexin repeats, folded into a domain that allows transient alignment with membrane lipids (16). Annexin a2 shares common features with other annexin family members, such as binding to anionic phospholipids in a calcium-dependent manner (17, 18). However, annexin a2 is distinguished from other annexins by its regulation of (19, 20) and interaction with the annexin a2 protein (21, 22). This complex, expressed on vascular endothelial cells, serves as a receptor platform for multiple proteins that differentially regulate wound healing processes, such as fibrinolysis and vascular invasion through the extracellular matrix (23–32).

For A2t-mediated vascular fibrinolysis and endothelial cell invasion, it is generally accepted that both the p11 and annexin a2 proteins are required, although evidence for a direct role of annexin a2 to activate these processes has been contested (33–36). Supporting the requirement for both proteins, mice deficient in either annexin a2 or p11 protein expression have defective fibrin clearance and angiogenesis (29, 37). However, it is important to note that annexin a2 protein expression has been shown to regulate the stability of p11 protein in vitro (20, 38, 39) and in vivo (19), therefore making it difficult to interpret which protein deficit was responsible for the observed phenotype. Subsequently, the A2t ligand, plasminogen, was found to bind to monomeric p11 protein but not monomeric annexin, bolstering evidence that p11 might be the effector protein for A2t-mediated fibrinolysis (23). Regardless, annexin a2 appears to be required to stabilize and localize p11 to the plasma membrane (40), where the heterotetramer contributes to remodeling of the extracellular matrix in the vasculature.

Membrane phospholipids may differentially regulate A2t function, stability, or localization (41). In previous reports, the heterotetramer demonstrated limited interaction with phosphatidylcholine but associated readily with a mixture of anionic lipids containing phosphatidylserine (23) or phosphoinositides (42). In contrast, monomeric p11 (not the heterotetrameric form associated with annexin a2) did not interact with phospholipids (23). Both electron microscopy and scanning force microscopy have revealed images of annexin a2 positioned as a bridge between p11 and the plasma membrane (43, 44), arguing that the annexin moieties may function to localize p11 to the plasma membrane. It is unknown how the interaction of A2t with membrane phospholipids might affect protein function. Our goal was to elucidate whether the lipid microenvironment, and C-1-P enrichment in particular, would alter A2t activity. Additionally, we sought to identify effector proteins that mediated chemotaxis by C-1-P. Because annexin a2 has an affinity for anionic phospholipids (like C-1-P), we investigated the role of sphingolipids in the A2t-mediated vascular wound healing response of endothelial cell invasion.

**EXPERIMENTAL PROCEDURES**

**Materials**—All lipids were purchased from Avanti Polar Lipids (Alabaster, AL) and were as follows: ceramide, N-palmitoyl-d-erythro-sphingosine; PA, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate; C-1-P, N-palmitoyl-ceramide 1-phosphate; S-1-P, d-erythro-sphingosine 1-phosphate; DOPS, 1,2-dioleoyl-sn-glycero-3-phospho-serine; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine. BSA was purchased from EMD Millipore (Rockland, MA). Other chemicals were purchased from Sigma, unless indicated otherwise. For Western blotting, p11 (ab94348) and annexin a2 (ab41803) antibodies were purchased from Abcam (Cambridge, MA), and β-actin antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). siRNAs were purchased from Invitrogen. The ceramide kinase gene, prepared from HUVEC cDNA, was cloned into the pcDNA 3.1 vector, from Invitrogen. HPA sensor chips were from GE Healthcare.

**Cell Culture**—Primary human retinal microvascular endothelial cells (HREC) and primary human coronary artery endothelial cells (HCAEC) were maintained, according to instructions provided by the supplier, in their specified media (Cell Systems Corp. (Kirkland, WA) and Cell Applications, Inc. (San Diego, CA), respectively). HREC were maintained in phenol red-free medium with 10% serum and CultureBoost™ (Cell Systems Corp.). When conducting serum starvation, HREC were maintained in basal medium without serum, CultureBoost™, or other supplementation, and HCAEC were serum-starved in basal medium without growth supplementation. All cells were incubated at 37 °C in 5% CO₂. For all experiments, HREC did not exceed passage 9, and HCAEC did not exceed passage 15.

**Preparation of Lipids in BSA Vehicle**—Lipids were dried under a stream of nitrogen gas and then resuspended in DMSO. The lipid/DMSO solution was then added to a fatty acid-free medium with 10% serum and CultureBoost™ (Cell Systems Corp.). When conducting serum starvation, HREC were maintained in basal medium without serum, CultureBoost™, or other supplementation, and HCAEC were serum-starved in basal medium without growth supplementation. All cells were incubated at 37 °C in 5% CO₂. For all experiments, HREC did not exceed passage 9, and HCAEC did not exceed passage 15.

**Preparation of Lipids in BSA Vehicle**—Lipids were dried under a stream of nitrogen gas and then resuspended in DMSO. The lipid/DMSO solution was then added to a fatty acid-free BSA solution to achieve a final concentration of 1 mM lipid, 1 mM BSA in 20 mM HEPES with 10% DMSO. Complexes were allowed to form by rocking at room temperature for 30 min, followed by sonication until clarity improved. Lipids in BSA vehicle were stored at −20 °C, and multiple freeze/thaw cycles were avoided.

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BrdU Cell Proliferation Assay—Cells were seeded at a density of 20,000 cells/well into 96-well plates, followed by serum starvation overnight prior to treatment. Cells were treated with drug or lipid for 24 h. BrdU labeling lasted for 24 h concurrent with the treatment regimen. BrdU incorporation into DNA was measured with the colorimetric BrdU cell proliferation ELISA (Roche Applied Science) and was performed according to the manufacturer’s instructions.

Migration Assay—Cell migration was measured with the collagen-coated Oris™ cell migration assay (Platypus Technologies, Madison, WI), according to the manufacturer’s instructions. 50,000 cells/well were seeded in serum-free medium into the stoppered, collagen-coated 96-well plates and allowed to adhere for at least 4 h. Stoppers were then removed, and cell treatments were applied for 18–24 h. For detection of migrated cells, cells were washed with Hanks’ buffered salt solution with calcium and magnesium, followed by calcein AM fluorescence staining for 30 min.

Transfection—HREC and HCAEC were electroporated with the Amaza Nucleofector II instrument, following Amaza HCAEC kit instructions, using protocol S-005, with 0.5 × 10^6 cells and either 2 µg of plasmid DNA or 25 pmol of siRNA. Cells were allowed to recover for 48–72 h prior to further experimentation.

Western Blotting—Cells were rinsed with ice-cold PBS and scraped into lysis buffer composed of 50 mM HEPES, pH 7.5, 137 mM NaCl, 2 mM NaVO₄, 10 mM Na₃P₂O₇, 1% Nonidet P-40, 10% glycerol, 50 mM NaF, 1 mM EGTA, 2 mM EDTA, 2 mM β-glycerophosphate, 10 mM benzamidine, 2 mM PMSF, and protease inhibitor mixture (Roche Applied Science). Lysates were incubated on ice for 20 min and centrifuged at 14,000 g for 10 min, and supernatant was retained. Samples were loaded for 4–12% gradient NuPAGE precast gels (Invitrogen), and proteins were transferred to nitrocellulose (GE Healthcare). Blots were incubated overnight at 4 °C, and then washed five times with PBS. However, high background binding of C-1-P to these beads precluded their use for C-1-P measurements, so we instead coupled normal mouse IgG and p11 antibodies (ab89438, Abcam) was rotated with GammaBind™ G beads (GE Healthcare) at 4 °C for 6–18 h, and the beads were washed three times with PBS. However, high background binding of C-1-P to these beads precluded their use for C-1-P measurements, so we instead coupled normal mouse IgG and p11 antibodies to Dynabeads (Invitrogen), following the manufacturer’s instructions, to measure p11-associated C-1-P. Cells were lysed by sonication in a detergent-free buffer that consisted of 50 mM Tris-Cl, pH 7.5, 280 mM NaCl, 0.5 mM EDTA, 1 mM PMSF, and protease inhibitor mixture (Roche Applied Science). Cell lysate was added to antibody/bead slurry, rotated overnight at 4 °C, and then washed five times with PBS. Immunoprecipitation of p11 was verified by silver staining, following the kit instructions (Pierce), or Western blotting.

Lipid Extraction and Measurement by Mass Spectrometry—Sphingolipids from cell lysates and immunoprecipitations were extracted and measured by mass spectrometry on an AB Sciex 4000 Q Trap® LC/MS/MS (Framingham, MA) instrument as described previously (10). Phosphoglycerolipids and sphingolipids from conditioned medium were extracted as described previously (45) by methyl-tert-butyl ether extraction and analyzed on an AB Sciex TripleTOF® 5600 instrument. Peak areas for lipids measured were compared with those of internal standards. All data reported are based on monoisotopic mass and are represented as pmol/mg protein. For lipid extractions following immunoprecipitation of p11, lipid mass was normalized to the total mass of protein immunoprecipitated.

Lipid Binding A2t ELISA—Lipids were bound to Polysorp Immunoplates (Nunc, Rochester, NY) overnight at 25 °C, and 1.67 nmol of lipid was added to each well in 100 µl of TBS. After five washes with TBS, wells were blocked with 1% BSA in TBS for 1 h. Purified bovine A2t (GenWay Biotech, San Diego, CA), diluted in TBS with 10 mM CaCl₂, was incubated in the lipid-coated wells for 2 h, followed by three washes of TBS or of TBS + 10 mM CaCl₂, as indicated (for calcium-dependent binding, all subsequent washes and incubations were performed...
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with the addition of 10 mM CaCl₂). Antibodies for p11 or annexin a2 were added to wells at a 1:5000 dilution for 1 h. After three washes, HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.) were added to each well at a 1:2000 dilution. Bound antibody was detected with o-phenylenediamine dihydrochloride (Sigma) at 492 nm.

**Invasion Assay**—Cell invasion through BD Matrigel™ was measured using the BD Biocosciences endothelial cell invasion kit with a 3-µm Fluoroblok™ membrane 24-well plate, following instructions from the manufacturer. Alternatively, the Fluoroblok™ plates were purchased and coated with 100 µl of 300 µg/ml BD Matrigel™ diluted and prepared according to the manufacturer’s instructions. For HREC, 125,000–250,000 cells were seeded in each well; for HCAEC, 50,000 cells were seeded per well. Calcein AM labeling in Hanks’ buffered salt solution was used to detect cells that had invaded through the Matrigel™ after 24 h on a Molecular Devices Flex Station 3 plate reader (Sunnyvale, CA).

**Statistical Analyses**—Data are presented as means ± S.E. Statistics were performed using GraphPad Prism version 5.01 software utilizing one-way analyses of variance with the Tukey post-test or Student’s t test as appropriate.

**RESULTS**

To examine the role of exogenous C-1-P to mediate annexin a2-p11 heterotetramer function in the vasculature, all studies were conducted in HREC and HCAEC. Because C-1-P is considered a promigratory sphingolipid (1–3), we initially hypothesized that C-1-P would mediate cellular invasion and migration through an interaction with the annexin a2-p11 heterotetramer. We first assessed whether C-1-P could initiate chemotaxis of endothelial cells through Matrigel™, a basement membrane matrix preparation. Indeed, we found that exogenous C-1-P dramatically enhanced the capacity of both cell lines to invade through Matrigel™ in a dose-dependent manner, up to 10 µM in HREC and 3 µM in HCAEC (Fig. 1, A and B). Further, in HCAEC, the number of cells induced to invade the barrier by exogenous 3 µM C-1-P exceeded the number of cells induced to invade by complete medium containing serum, which contains multiple growth factors (Fig. 1B).

To explore the specificity of C-1-P-mediated invasion, we tested whether other lipids, such as the structurally similar sphingolipids ceramide and sphingosine 1-phosphate (S1-P), or the anionic phosphoglycerolipids PA and DOPS, which are known to interact with A2t (17, 23), could direct invasion in endothelial cells. We found that in HREC, 3 µM C-1-P dramatically promoted invasion compared with a BSA vehicle control, whereas 3 µM PA produced comparably moderate chemotaxis (Fig. 1C). In HCAEC, only 3 µM C-1-P induced statistically significant chemotaxis (Fig. 1D). Overall, these data suggest that 3 µM C-1-P selectively stimulates macrovascular and microvascular endothelial cell invasion through Matrigel™.

We also investigated the contribution of prostanooid production to the induction of endothelial invasion by C-1-P. C-1-P is a positive regulator of cytosolic phospholipase A₂ (cPLA₂), enhancing the membrane localization and activity of this rate-limiting enzyme in prostanooid production (7–9). To elucidate a contribution by prostanooids, we monitored C-1-P-mediated invasion in the presence of two cyclooxygenase inhibitors, acetylsalicylic acid (ASA) or celecoxib, which limit prostanooid production downstream of cPLA₂. In HREC, neither ASA nor celecoxib could inhibit C-1-P-mediated chemotaxis (Fig. 1E). Instead, in HREC, celecoxib augmented C-1-P-mediated invasion. In HCAEC, ASA had no effect on C-1-P-mediated invasion (Fig. 1F). We did detect a decrease in C-1-P-mediated invasion with celecoxib (Fig. 1F), indicating that prostanooids may play a minor role in C-1-P-mediated invasion in HCAEC. In total, however, these data suggest that C-1-P-induced endothelial cell invasion is not primarily mediated via prostanooid synthesis.

We next measured the contribution of migration and proliferation in C-1-P-mediated endothelial cell invasion. Because C-1-P substantially promoted invasion through an extracellular matrix-like barrier, it was consistent that 3 µM C-1-P promoted cellular migration over a collagen substrate in HREC and HCAEC when compared with a BSA vehicle control (Fig. 2, A and B). When we assessed the capacity of C-1-P to induce endothelial cell proliferation, we found that, in agreement with previous work from our laboratory (46), 3 µM C-1-P did not increase endothelial cell proliferation as measured by BrdU incorporation (Fig. 2, C and D). Although C-1-P treatment induces cell proliferation and migration in many cell types (1, 47–52), cell proliferation did not contribute to C-1-P-mediated invasion or migration in HREC or HCAEC.

To monitor sphingolipid and phospholipid metabolism following exogenous delivery of C16 C-1-P, we extracted sphingolipids from cell lysates at various time points and quantified them by mass spectrometry. In HREC and HCAEC, treatment with exogenous C16 C-1-P resulted in a dramatic increase in C16 C-1-P mass in cell lysates after 1 h of treatment (Fig. 3, A and B). Further increase in C-1-P mass was detected at 8 and 24 h in HREC, whereas C16 C-1-P mass in HCAEC was stable over time, alluding to a difference in the delivery kinetics between these two cell lines. In support of C16 C-1-P as the effector lipid throughout this report, the mass of other C-1-P species did not significantly change after exogenous treatment of C16 C-1-P; nor was there any significant change in phosphatidylserine mass or of phosphatidylserine fatty acid composition (data not shown).

All experiments in this work utilized C16 chain length C-1-P. To demonstrate the physiological relevance of our experimental design, we monitored the contribution of C16 C-1-P to total C-1-P mass, endogenous C-1-P production, and extracellular C-1-P. As measured by mass spectrometry, total C-1-P mass from untreated HREC cell lysates consisted of 55% C16 C-1-P, 28% C18 C-1-P, 11% C24:0 C-1-P, and 6% C24:1 C-1-P (data not shown). Likewise, in HCAEC cell lysates, C-1-P species consisted of 89% C16 C-1-P, 3% C18 C-1-P, 7% C24:0 C-1-P, and 1% C24:1 C-1-P (data not shown). Thus, the most prevalent endogenous species in both HREC and HCAEC is C16 C-1-P.

To confirm that ceramide kinase-derived C-1-P is indeed found in extracellular space and available to act on extracellular receptors, we transfected HREC with an empty vector or ceramide kinase expression vector to measure intracellular and extracellular C-1-P (Fig. 3, C and D). To monitor extracellular C-1-P, we measured C-1-P mass in conditioned medium (co-
lected and spun at 3000 × g for 10 min to remove cellular debris) and compared that with C-1-P mass from cell lysates 48 h following transfection. We found that empty vector- and ceramide kinase-transfected HREC were exposed to 2- and 5-fold more extracellular C-1-P mass, respectively, than the mass of C-1-P contained within the cell lysate fraction. This demonstrated that C-1-P was readily available to act on extracellular receptors on endothelial cells. Ceramide kinase transfection led to a significant increase in both extracellular and intracellular C-1-P in HREC when compared with a basal level in empty vector-transfected cells, demonstrating that ceramide kinase overexpression leads to an increase in total C-1-P mass and the amount of C-1-P mass found extracellularly.

To demonstrate that extracellular C-1-P could bind to A2t and its protein constituents, we employed surface plasmon resonance, immunoprecipitation, and lipid-binding ELISA strate-
gies. Specifically, surface plasmon resonance was employed to measure the lipid binding affinity and selectivity of annexin a2 for anionic lipid-containing membranes. All measurements were conducted in the presence of calcium. Using the HPA sensor chip, an adsorbed monolayer of PC/PE (80:20) was used as a control surface on flow channel 1, whereas flow channel 2 was used for the active surface, which consisted of PC/PE/x (70:20:10), where x represents C-1-P, PS, or PA. Five or more concentrations of annexin a2 (from 1 to 800 nM) were injected to obtain RU saturation values at each respective concentration of annexin a2. Saturating RU signals were then plotted versus annexin a2 concentration to obtain an equilibrium binding curve, which was fit with a nonlinear least squares analysis of the binding isotherm to determine the $K_d$. The results (Fig. 4) demonstrate that the annexin a2 protein binds to 10 mol% C-1-P membranes with the highest affinity, 11 nM (Fig. 4E), which was 17- and 14-fold stronger than binding to PS and PA vesicles, respectively.

Next, we tested the hypothesis that C-1-P interacted with the p11 protein component of A2t. We treated HREC or HCAEC with 3 μM lipids or a BSA vehicle control for 10 min, immunoprecipitated p11 (Fig. 5, A and D), and then quantified the associated phosphoglycero- and sphingolipids by mass spectrometry. Exogenous C-1-P treatment dramatically increased the amount of C16 C-1-P associated with p11 protein when compared with a C-1-P-treated sample immunoprecipitated with a nonspecific IgG control or a BSA vehicle-treated sample immunoprecipitated with p11 (Fig. 5, B and E). We found that exogenous DOPS treatment did not alter p11-associated DOPS mass when compared with a PA-treated control (Fig. 5, C and F), although it should be noted that DOPS did bind annexin a2 in surface plasmon resonance experiments (Fig. 4), albeit with lower affinity than C-1-P. We also detected strong binding of PA to p11 in HCAEC (Fig. 5G). Despite readily detecting a PA internal standard in HREC lipid extracts, demonstrating that our method was robust, we did not detect any p11-associated PA in HREC (data not shown). Overall, these data support a C-1-P/p11 interaction. The data further indicate that exogenously delivered C-1-P was enriched in p11 protein complexes in two independent cell lines. Although we were able to successfully immunoprecipitate p11 protein (Fig. 5, A and D), repeated attempts to immunoprecipitate annexin a2 were confounded by strong binding of annexin a2 protein to normal, nonspecific IgG antibody controls (data not shown). This may be because annexin a2 is posited to bind to and function as an IgG transporter in the placenta (53).

After measuring binding of annexin a2 to lipids via surface plasmon resonance (Fig. 4) and measuring the p11 interactions with lipids by immunoprecipitation and mass spectrometry (Fig. 5), we wanted to provide confirmation that C-1-P could directly associate with the A2t heterotetrameric protein complex. To accomplish this, we measured the relative binding of lipids to purified bovine A2t by utilizing a lipid-binding ELISA strategy (Fig. 6). Results were verified by independently testing...
both a p11 and annexin a2 antibody for detection of a positive interaction. Detection with the p11 antibody (Fig. 6A) and the annexin a2 antibody (Fig. 6B) generated similar results and served to confirm that the A2t heterotetrameric protein complex remained stable during the experiment. As expected, A2t demonstrated strong binding to DOPS and PA, because these interactions have been previously characterized in cell-free systems (17, 23) and in our surface plasmon resonance experiment (Fig. 4). We also detected binding of A2t to C-1-P. To confirm that this assay detected C-1-P binding to A2t with high specificity, we performed all steps of the ELISA, with the exclusion of A2t protein, in C-1-P-coated wells. No measurable signal was detected in these wells, eliminating any concern that C-1-P interacted nonspecifically with any of the ELISA antibodies or reagents. No significant binding of A2t to ceramide or DOPC was detected, suggesting a requirement for negatively charged lipids. Surprisingly, no measurable interaction between A2t and S-1-P was detected, suggesting that two hydrophobic chains may be required for a lipid interaction with A2t. In the next series of experiments, we explored the role of calcium in lipid binding to A2t. Because the binding of A2t to PA and DOPS is calcium-dependent (17), we measured a requirement for calcium in the interaction of C-1-P and A2t. C-1-P, like PA and DOPS, required calcium to bind A2t (Fig. 6C).

Next, we sought evidence that C-1-P-mediated invasion in endothelial cells required A2t. To interfere with A2t expression, we disrupted p11 or annexin a2 protein expression through siRNA-mediated knockdown (Fig. 7, A and B). In HREC, knockdown of annexin a2 led to decreased p11 protein expression (Fig. 7A), consistent with reports in the literature that annexin a2 regulates p11 expression (19, 20, 38, 39). In contrast, the modest knockdown of annexin a2 achieved in HCAEC (38% knockdown when compared with negative control siRNA) did not decrease p11 protein expression in this endothelial cell line (Fig. 7B), suggesting cell-specific differences in A2t regulation. Regardless, we assessed the ability of annexin a2- or p11-depleted cells to invade across a Matrigel™ barrier. HREC with decreased p11 protein expression were significantly less responsive to C-1-P-mediated invasion when compared with the negative control siRNA-transfected HREC (Fig. 7C). Likewise, transfection of HREC with annexin a2 siRNA, which decreased both annexin a2 and p11 expression, significantly reduced C-1-P-mediated invasion (Fig. 7C). In HCAEC, knockdown of either p11 or annexin a2 significantly decreased C-1-P-mediated invasion through Matrigel™ (Fig. 7D). In sum, C-1-P-mediated invasion in vascular endothelial cells is dependent on expression of either p11 or annexin a2 proteins, implicating a dependence on the A2t heterotetrameric protein.

**DISCUSSION**

We have now demonstrated that C-1-P interacts directly with A2t in both macro- and microvascular endothelial cells to mediate invasion across an extracellular matrix barrier. Through surface plasmon resonance, we determined that 10 mol % C-1-P-containing membranes selectively bind annexin a2 and A2t.
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A2 protein at $K_d = 11$ nm (Fig. 4). By immunoprecipitating p11 protein and identifying associated lipids, we also demonstrated an interaction between C-1-P and p11 in endothelial cells (Fig. 5). Because this finding implied an interaction with A2t but did not rule out direct binding to monomeric p11 or another p11-protein complex interaction, we sought additional confirmation of an interaction between C-1-P and the A2t heterotetramer. Therefore, binding to the A2t complex was confirmed with a lipid-binding ELISA utilizing the purified annexin a2-p11 heterotetrameric protein complex (Fig. 6). Additionally, we demonstrated a requirement for both A2t constituents, the p11 and annexin proteins, in mediating chemotaxis by exogenous C-1-P through an siRNA-mediated knockdown strategy (Fig. 7).

In support of C16 C-1-P as the causative lipid in inducing endothelial cell invasion, we found that exogenous administration of C16 C-1-P led to a dramatic and prolonged increase in cellular C16 C-1-P mass (Fig. 3). Further, in these experiments, no significant changes in the cellular mass of other sphingolipids or phospholipids were detected (data not shown).

Our finding that A2t mediates cellular invasion by C-1-P (Figs. 1 and 7) is the first report to link binding of extracellular C-1-P with an identifiable cell surface protein receptor. Although A2t does not exclusively bind to C-1-P (Fig. 6), C-1-P significantly stimulated A2t-mediated cell invasion in HREC and HCAEC endothelial cell lines (Fig. 7). In contrast, of the panel of lipids with similar structural characteristics that we tested, PA, which also bound A2t (Figs. 4 and 6), only induced limited invasion in HREC relative to C-1-P (Fig. 1C). DOPS, which also bound A2t (Figs. 4 and 6), had no effect on endothelial cell invasion (Fig. 1C and D). We did note a slight induction of invasion by 3 μM S-1-P in HCAEC (Fig. 1D), and although it did not achieve statistical significance, further examination with reduced concentrations of S-1-P may reveal bioactivity to induce endothelial cell invasion. It has been reported previously that S-1-P utilized annexin a2 protein as an effector of endothelial cell invasion. Figure 4. Annexin A2 selectively binds to 10 mol % C-1-P-containing membranes as compared with other anionic lipids. By implementing surface plasmon resonance, equilibrium binding constants were determined in triplicate on a supported monolayer surface containing PC/PE/x (70:20:10), where x represents C-1-P, PS, or PA, and subtracted from a control surface composed of PC/PE (80:20). Each replicate was determined by injecting increasing concentrations of annexin a2 over the supported monolayer, where each point represents a saturation point on the sensogram. $K_d$ values for each lipid at 1 mM Ca$^{2+}$ were determined by fitting 5 points with a nonlinear least squares analysis of the binding isotherm ($R_{max} = \frac{R_{max}/(1 + K_d/C)}{K_d}$). A, representative sensograms from the data showing PC/PE/C-1-P binding responses at the indicated concentrations of annexin a2. Equilibrium binding curves for PC/PE/C-1-P (B), PC/PE/PS (C), and PC/PE/PA (D). E, $K_d$ values determined from an average of three separate experiments ± S.D.
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Figure 5. C-1-P interacts with p11 in endothelial cells. Cell lysates were immunoprecipitated with a nonspecific normal mouse IgG control or p11 antibody, separated by PAGE, and silver-stained to verify successful immunoprecipitation of p11 in HREC (A). M, marker; IgG, nonspecific IgG control immunoprecipitation (IP); p11, p11 antibody immunoprecipitation; T, total cell lysate. For HCAEC, immunoprecipitation of p11 was confirmed by Western blot (D). HREC or HCAEC were treated for 10 min with a 3 μM concentration of the indicated lipid or equivalent volume of BSA vehicle, and cells were then collected in a detergent-free lysis buffer and immunoprecipitated with normal mouse IgG or p11 antibody. After washing, the immunoprecipitation beads were added to 2:1 methanol/chloroform, followed by sphingolipid extraction and mass spectroscopy quantification to measure associated C-1-P in HREC (B) and HCAEC (E). For measurement of DOPS (C and F) and PA (G) by mass spectroscopy, beads were added to 1:3.3 water/methyl-tert-butyl ether, followed by phospholipid extraction. Note that we did not detect any PA associated with p11 in HREC cells. Shown is pmol of lipid/mg of protein input/immunoprecipitation, and, as such, data should be interpreted as -fold increase as compared with IgG control rather than as absolute values. *, p < 0.05; **, p < 0.01; ***, p < 0.001. Error bars, S.E.

Lial cell invasion in human umbilical vein endothelial cells (54). Our data do not, however, implicate S-1-P as a high affinity interactor with the heterotetrameric form of annexin a2-p11, because we found no binding of S-1-P to the A2t protein in the lipid-binding ELISA (Fig. 6) and no significant changes in S-1-P associated with p11 following immunoprecipitation (data not shown).

Induction of C-1-P-mediated invasion was dramatically inhibited when expression of p11 or annexin a2 was knocked down by siRNA technology (Fig. 7), indicating involvement of A2t. Because C-1-P potentiates cPLA2 activity (6–9), we examined the contribution of cPLA2 activation to C-1-P-mediated invasion. We found that cyclooxygenase inhibition by acetylsalicylic acid or celecoxib, which blocks prostanoid production, did not dramatically limit chemotaxis induced by C-1-P (Fig. 1, E and F). These data do not support a prominent role for cPLA2 in C-1-P-mediated endothelial cell invasion.

This report documents the binding of C-1-P to annexin a2, one of a few newly discovered C-1-P binding proteins (such as cPLA2 and TNF-α-converting enzyme) (6–9, 11). Unlike the binding of cPLA2 to C-1-P, which has been described as a coincidence detector for C-1-P and zwitterionic membranes (55), annexin a2 binds with very strong affinity (in the low nanomolar range) to membranes containing 10% C-1-P, further sup-
FIGURE 6. C-1-P interacts with A2t proteins. C-1-P binds the heterotetrameric protein complex in a cell-free assay. ELISA plates were coated with 1.67 nmol of the indicated lipid and incubated with purified bovine A2t with buffer containing 10 mM CaCl₂, followed by detection of a lipid-protein interaction with p11 (A) or annexin a2 (B) antibodies. Binding of A2t to C-1-P was calcium-dependent. When the assay was performed in the absence of CaCl₂, little binding to any lipid was detectable (C). Shown are relative binding data, represented by absorbance, with background subtracted. No A2t, no heterotetramer added to wells. Cer, ceramide. *, p < 0.05; **, p < 0.01; ***, p < 0.001 when compared with no A2t in A and B or when compared with no CaCl₂ in C. One representative experiment, of at least two independent experiments, is shown. Error bars, S.E.

FIGURE 7. Each constituent of the annexin a2-p11 heterotetramer is required for endothelial cell invasion mediated by C-1-P. HREC or HCAEC were transfected with negative control, p11, or annexin a2 (ANX A2) siRNAs. Cells were collected 48–72 h later for protein isolation or invasion assays. Protein knockdown in HREC (A) or HCAEC (B) was confirmed by Western blotting with p11 and ANX A2 antibodies. To monitor A2t-dependent invasion, siRNA-transfected HREC (C) or HCAEC (D) were treated with 10 or 3 μM C-1-P, respectively, and monitored for invasion through Matrigel™. Error bars, S.E. of at least three replicates. *, p < 0.05; **, p < 0.01; ***, p < 0.001 when compared with the negative control siRNA-transfected cells. #, p < 0.05; ##, p < 0.01; ###, p < 0.001 when compared with an equivalent volume of BSA vehicle per siRNA transfected.
porting the hypothesis that the annexin a2-p11 heterotetrameric protein complex may function as an extracellular C-1-P “receptor.” Further supporting this possibility, the affinity of annexin a2 for C-1-P was 14-fold greater than that of PA. One explanation for this difference could be the physical state of the membrane. C-1-P, for example, has been shown to induce domain formation in vesicles (57). C-1-P and PA also have different biophysical properties in membranes that may explain the difference in selectivity. First, C-1-P and PA have different pK_a values of the phosphomonoester headgroup (57, 58) that may elicit a larger anionic charge on C-1-P (~2) in DOPE and DOPC membranes. Additionally, 0.5–1 Ca^{2+} ions have been found to bind to each C-1-P molecule in a model membrane (59), which could suggest a Ca^{2+}-bridging model between annexin a2 and C-1-P in membranes.

We have presented evidence that C-1-P potently induced macro- and microvascular endothelial cell invasion and migration but not proliferation (Fig. 1 and 2), implying that C-1-P may promote positive remodeling during vascular wound healing. During arterial wound healing following angioplasty, differential ceramide kinase activity in endothelial cells and smooth muscle cells may have enabled ceramide-coated balloon catheters to promote re-endothelialization while preventing restenosis due to undesirable smooth muscle cell proliferation (46). We hypothesize that C-1-P-mediated suppression of proinflammatory cytokine release (10–12) during endothelial cell invasion may also control inflammation and limit restenosis during vascular wound healing, and this is an area of future investigation.

Although it is possible that intracellular accumulation of C-1-P after exogenous administration plays a role in C-1-P-stimulated invasion, we believe that extracellular C-1-P (Fig. 3), acting via extracellular A2t, fits into an accumulating body of work indicating that extracellular C-1-P gradients create spatial maps guiding cellular navigation. In RAW 264.7 cells, a mouse macrophage-like cell line, exogenous C-1-P acted as a chemotacticant, inducing cell migration through a G-protein-coupled receptor (1). In Drosophila, C-1-P gradients in the developing embryo regulate dorsal/ventral patterning by counteracting Wnt inhibitor of dorsal (WntD) signaling (3). Further, Drosophila double mutants with disrupted ceramide kinase and multistructure lipid kinase activities (the only enzymes known to phosphorylate ceramide) displayed defective migration of primordial germ cells to the gonads (3). S-1-P morphogenetic gradients (generated by endothelial cell eflux (60)) regulate the egress of lymphocytes from lymphoid tissues to peripheral blood (61). In like fashion, extracellular C-1-P can direct the navigation of circulating cells, such as hematopoietic stem/progenitor cells (HSPC). HSPC are guided to mobilize from or home to the bone marrow and circulatory system through peptides, such as stromal derived factor-1 (SDF-1), and sphingolipids such, as S-1-P and C-1-P (4). Specifically, S-1-P opposes the action of SDF-1, the prototypical HSPC retention signal, to facilitate HSPC egress from the bone marrow (4, 62–64). In contrast, C-1-P, in combination with SDF-1, acts as a potent homing factor for HSPC to return to the bone marrow from circulation (2). Notably, C-1-P mass increases in the bone marrow following irradiative myeloablation and recruits transplanted hematopoietic stem cells to the bone marrow for engraftment in a protease-rich environment hostile to proteins and peptides, such as SDF-1 (2). Lipid phosphate phosphatases, promiscuous enzymes that can dephosphorylate C-1-P, S-1-P, phosphatidic acid, and lysophosphatidic acid, terminate bioactive lipid gradient signals (65). This termination mechanism further supports the concept that bioactive sphingolipid gradients are true signals that can direct cells to navigate or home to different environments. Extracellular C-1-P released after vascular wounding may be a non-inflammatory mechanism for vascular progenitor cell recruitment to the wounded region and is an area for future investigation. Identification of A2t as an extracellular C-1-P-interacting protein further supports the possibility that sphingolipid gradients guide cellular migration and invasion.

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