Distinct signals via Rho GTPases and Src drive shape changes by thrombin and sphingosine-1-phosphate in endothelial cells

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Summary

Soluble mediators such as thrombin and sphingosine-1-phosphate regulate morphological changes in endothelial cells that affect vascular permeability and new blood vessel formation. Although these ligands activate a similar set of heterotrimeric G proteins, thrombin causes cell contraction and rounding whereas sphingosine-1-phosphate induces cell spreading and migration. A functional requirement for Rho family GTPases in the cytoskeletal responses to both ligands has been established, yet the dynamics of their regulation and additional signaling mechanisms that lead to such opposite effects remain poorly understood. Using a pull-down assay to monitor the activity of Rho GTPases in human umbilical vein endothelial cells, we find significant temporal and quantitative differences in RhoA and Rac1 activation. High levels of active RhoA rapidly accumulate in cells in response to thrombin whereas Rac1 is inhibited. In contrast, sphingosine-1-phosphate addition leads to comparatively weak and delayed activation of RhoA and it activates Rac1. In addition, we show here that sphingosine-1-phosphate treatment activates a Src family kinase and triggers recruitment of the F-actin-binding protein cortactin to sites of actin polymerization at the rim of membrane ruffles. Both Src and Rac pathways are essential for lamellipodia targeting of cortactin. Further, Src plays a determinant role in sphingosine-1-phosphate-induced cell spreading and migration. Taken together these data demonstrate that the thrombin-induced contractile and immobile phenotype in endothelial cells reflects both robust RhoA activation and Rac inhibition, whereas Src- and Rac-dependent events couple sphingosine-1-phosphate receptors to the actin polymerizing machinery that drives the extension of lamellipodia and cell migration.

Key words: Rho proteins, Src, S1P, Thrombin, HUVEC, Cell migration

Introduction

Regulated assembly of the actin cytoskeleton in endothelial cells is required throughout the angiogenic process. Morphological changes underlie both the rapid breakdown of the endothelial permeability barrier and subsequent increases in endothelial cell migration. While cues for these effects come from cell-cell contacts and the extracellular matrix, diffusible factors generated by the hemostatic system are emerging as important modulators of angiogenesis (Browder et al., 2000; Carmeliet, 2001). Two such mediators, thrombin and sphingosine-1-phosphate (S1P), exert distinctly different effects on endothelial cell shape. To understand the mechanisms underlying these differences, we have analyzed the precise regulation of Rho family proteins by these ligands in human umbilical endothelial cells (HUVECs). Our studies have uncovered an important role for Rac and Src activation in S1P-induced spreading and migration.

The protease thrombin, a major effector of the coagulation cascade, induces cell contraction and rounding in cultured endothelial cell monolayers. This shape change is accompanied by increased stress fiber formation and has been shown to involve a Rho/Rho kinase-dependent pathway (Essler et al., 1998; Hippenstiel et al., 1997; Vouret-Craviari et al., 1998). Previous studies on an immortalized endothelial cell line (Vouret-Craviari et al., 1998) and, more recently, on primary cultured HUVECs (Wojciak-Stothard et al., 2001) have suggested that Rac also participates in controlling the cytoskeletal effects of thrombin. Thrombin channels information for its cellular actions through protease-activated receptors (PARs) coupled to heterotrimeric G proteins. Two of the three thrombin-responsive PARs, PAR-1 and -3, are expressed by HUVECs (Molino et al., 1997; Schmidt et al., 1998), yet cytoskeletal reorganization by thrombin in these cells appears to be mediated by PAR-1 (Vouret-Craviari et al., 1998).

S1P is a lysophospholipid stored and released by activated blood platelets. This lipid mediator initiates a variety of responses in endothelial cells including the stimulation of proliferation, survival and morphogenesis. Although some biological activities of S1P have been reported to be mediated through intracellular targets, it is becoming increasingly clear that the above-mentioned S1P-induced cellular responses are mediated by G-protein-coupled receptors of the endothelial differentiation gene (EDG) family (for reviews, see Hla et al., 2000; Pyne and Pyne, 2000; Spiegel and Milstien, 2000). Among the five S1P-specific receptors identified to date, only EDG-1 and EDG-3 are expressed by HUVECs, with levels of EDG-1 being significantly higher than EDG-3 in these cells (Lee, O. H. et al., 1999).

Several studies over the past two years have documented the potent chemotactic activity of S1P for vascular endothelial cells (Lee et al., 2000; Lee, M.-J. et al., 1999; Paik et al., 2001; Panetti et al., 2000; Wang et al., 1999). Cell migration is a complex process that requires the polymerization of actin filaments behind the leading edge of the cell to drive the extension of lamellipodia. Recently, a novel role for the F-actin-binding protein, cortactin, has been established in the formation of membrane ruffles. Cortactin (p80/p85) was first discovered as the major phosphotyrosine-containing protein in v-Src-transformed fibroblasts (Wu and Parsons, 1993) and subsequently described as an oncogene frequently amplified in tumors and tumor cell lines (Schuuring et al., 1993); it was found to interact directly with the actin-related protein 2/3 (Arp2/3) complex at sites of actin polymerization within lamellipodia (Weed et al., 2000), where it stimulates its actin-nucleation activity and stabilizes the actin filament network formation (Uruno et al., 2001; Weaver et al., 2001). We show here that S1P induces selective targeting of cortactin to membrane ruffles, peripheral actin polymerization and migration in endothelial cells and we provide evidence for functional requirement of Src family kinases in these effects.

Materials and Methods

Materials

All reagents, unless specified, were from Sigma. Tissue culture plasticware was from Life Technologies. Human α-thrombin (3209 NIH units/mg) was kindly supplied by J. W. Fenton, II (New York State Department of Health, Albany, NY). Sphingosine-1-phosphate (S1P) was from BioMol Research Laboratory. The Rho kinase inhibitor (Y-27632) was kindly supplied by A. Yoshimura (Welfide Co, Japan) and the Src family kinase inhibitor, PP2 [4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazololo[3,4-d]-pyrimidine], was from Calbiochem. The MEK inhibitor (U0126) was from Promega.

Cell culture and electroporation conditions

HUVECs were isolated from umbilical cords as previously described (Barbieri et al., 1981) and cultivated on 0.2% gelatin-coated 10 cm diameter dishes in human SFM Medium (Life Technologies) supplemented with EGF (10 ng/ml), bFGF (20 ng/ml), heparin (100 μg/ml) and 20% heat-inactivated fetal calf serum (FCS, Life Technologies). Cells were maintained at 37°C in 5% CO_2 and used at the fifth passage for all experiments described herein. It is important to note that endothelial marker expression and agonist responsiveness of HUVECs cultured in SFM medium (compared with M199 plus 20% FCS, endothelial cell growth supplement and heparin) is comparable with that of earlier passage cells.

For gene expression, 60 μg of total DNA were added to 5×10^6 HUVECs and cells were subjected to electroporation (300 V, 450 μF). Cultures were allowed to recover overnight in 10 cm diameter gelatin-coated dishes before replating onto fibronectin-coated coverslips for analysis. After spreading, cells were serum-starved for 6 hours before agonist treatment.

Antibodies

26C4 mouse monoclonal anti-RhoA antibody was kindly supplied by J. Bertoglio (INSERM U461, Paris, France). Mouse monoclonal anti-Rac1 (clone 23A8) was from Upstate Biotechnology and the polyclonal anti Cdc42 was a generous gift of I. Just (Institute for Pharmacology and Toxicology, Freiburg, Germany). The anti-Src antibody (cst-1) used in this study was generously provided by S. Roche (CRBM, Montpellier, France). Cst-1 is an affinity-purified antibody that recognizes the C-terminal sequence of Src, Yes and Fyn proteins on immunoblots and in immunoprecipitation assays. The monoclonal anti-activated Erk (ERK-1 and ERK-2) antibody, clone MAPK-YT, was from Sigma. Rabbit polyclonal anti-pSrc (pY^{418}) and mouse monoclonal anti-cortactin (clone 4F11) antibodies were from BioSource International and Upstate Biotechnology, respectively. Anti-phosphotyrosine antibody (clone PY99) was purchased from Santa Cruz Biotechnology and we used a rabbit polyclonal anti-Myc tag antibody purchased from MBL (Japan). Secondary antibodies coupled to horseradish peroxidase or alkaline phosphatase were from Promega and New England Biolabs, respectively. Fluorescently-labeled secondary antibodies were purchased from Molecular Probes.

Determination of Rho protein activity

HUVEC cultures grown to confluence in 10 cm diameter dishes were starved overnight in human SFM medium. The RhoA assay was performed as follows. Stimulated cells were lysed in buffer A (25 mM Hepes pH 7.3, 150 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.5% Triton X-100, 4% glycerol, 20 mM β-glycerophosphate, 10 mM NaF, 2 mM sodium orthovanadate, 5 mM dithiothreitol and protease inhibitors) and incubated for 10 minutes at 4°C. Triton-X-100-insoluble material was removed by centrifugation (10 minutes, 9500 ***g***) and the lysates were incubated with 20 μg of bacterially produced GST-rhotekin (Ren et al., 1999) bound to glutathione-coupled Sepharose beads, for 40 minutes at 4°C. Beads were washed four times in buffer A, resuspended in loading buffer and proteins were separated by SDS-PAGE on 12% acrylamide gels prior to western blottting with anti-Rho antibody. We followed the same procedure for Rac1 and Cdc42 assays except that appropriate cellular lysates were incubated with 10 μg of bacterially produced GST-PAK (Bagrodia et al., 1995). Beads and bound proteins were then rinsed four times in lysis buffer, and Rac1 and Cdc42 were detected by western blotting. Prior to the incubation with the beads, 50 μl aliquots were removed from all samples to control for equal loading of total RhoA, Rac1 and Cdc42 proteins.

Immunofluorescence studies

HUVECs plated on fibronectin-coated glass coverslips were serum starved overnight and treated for the indicated times with agonists. To stop the reaction and fix the cells, a solution of 3% paraformaldehyde and 2% sucrose was added for 15 minutes at room temperature. After three washes in PBS, cells were permeabilized with 0.2% Triton X-100 for 3 minutes and stained with the indicated antibodies for 1 hour at room temperature in a humid chamber. When indicated, a wound (typically between 30 and 50 μm) was made by scraping confluent monolayers with a 26G needle. Fluorescence was observed with a Nikon Diaphot fluorescence microscope or a Leica TCSSP confocal laser scanning microscope.

Src kinase assay

Quiescent HUVECs were treated with agonists for the indicated times at 37°C and the Src autophosphorylation assay was performed as previously described (Chen et al., 1994). Briefly, Src proteins were immunoprecipitated with cst-1 antibody (4 μl/1.5 mg proteins) bound to protein A sepharose and kinase assays were carried out on ice for 15 minutes in the presence of (γ-^{32}P)ATP. Samples were analyzed by SDS-PAGE on 12% gels under reducing conditions. The gels were treated with 1 M KOH at 60°C for 1 hour and radioactivity was quantified using a Fuji phosphorimager.

Results

Rho GTPase activation by thrombin and S1P

We first set out to compare the precise temporal regulation of Rho, Rac and Cdc42 activity by thrombin and S1P in

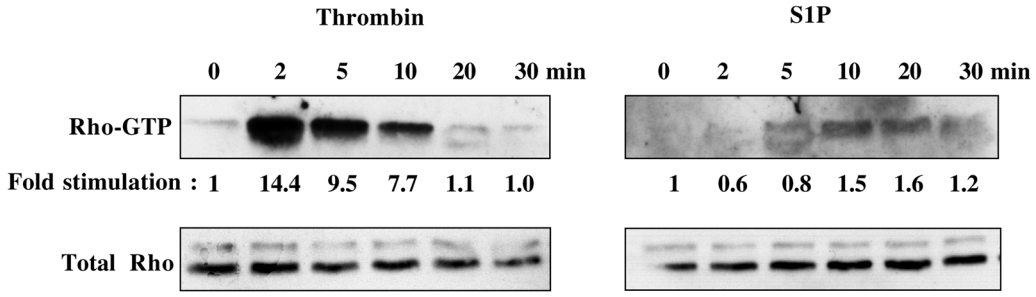

Fig. 1. Temporal activation of Rho A by thrombin and S1P. Confluent monolayers of HUVECs were cultivated as indicated in Materials and Methods and, after overnight starvation, were treated with thrombin (10 nM) or S1P (0.5 μM) for the times indicated. The amount of RBD-bound and total RhoA in cell extracts was determined by western analysis. A minor band, not regulated by agonists, was often detected above total RhoA, which likely corresponds to a geranyl-geranylated form of the protein. The results shown are representative of three independent experiments in which thrombin and S1P effects were directly compared. Fold stimulation of GTP-bound Rho was determined following normalization against total Rho.

HUVECs. The activation state of endogenous Rho proteins in agonist-stimulated cells was determined using GST-fusion proteins to specifically pull-down active forms of RhoA, Rac1 or Cdc42 from cell lysates. Given the high degree of sequence homology among these three GTPases, we first verified that each of the three antibodies used for detection was highly specific for its respective antigen (data not shown).

Fig. 1 shows the accumulation of active (GTP-bound) RhoA isolated with GST-rhotekin from lysates of HUVEC challenged with thrombin (left panel) or S1P (right panel). Total RhoA (GDP- plus GTP-bound) present in the corresponding lysates is depicted in the lower panels. As it can be seen here, 10 nM thrombin induces robust activation of RhoA. Accumulation of active RhoA in response to thrombin is rapid and transient. Maximal stimulation is observed by two minutes and the amount of Rho-GTP returns to basal levels after 20 minutes. In contrast, the activation of RhoA in response to 0.5 μM S1P is significantly weaker and delayed. This concentration of S1P was found to be maximally effective for cytoskeletal modifications and activation of the MAP kinase Erk 1/2 in our HUVEC cultures (data not shown). Clearly, these two agonists differ in their ability to activate RhoA in HUVECs.

It has previously been reported that the CRIB (Cdc42 and Rac interacting binding) domain of p21-activated kinase (PAK) interacts with active forms (GTP-bound) of both Rac and Cdc42 (Bagrodia et al., 1995). Using a GST-PAK fusion protein, active forms of Rac1 and Cdc42 were isolated from confluent HUVECs following different times of treatment with thrombin or S1P. As shown in Fig. 2, we were unable to detect any significant modulation of Cdc42 activity by thrombin or S1P (top panels). A time-dependent activation of Cdc42 could be observed in these cells following treatment with cytotoxic necrotizing factor 1, a Rho GTPase-activating toxin (data not shown). Interestingly, thrombin and S1P exert divergent effects on Rac1 activity. To our surprise, thrombin was found to inhibit basal Rac1 activity. This inhibition occurs shortly after thrombin addition and slowly reverses to reach near-basal

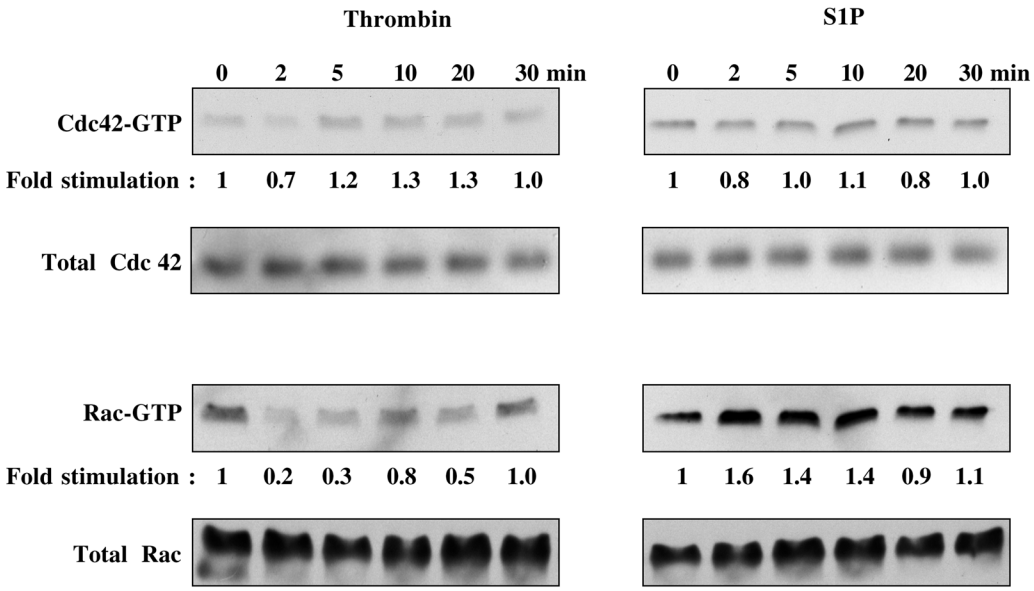

Fig. 2. Temporal activation of Cdc42 and Rac1 by thrombin and S1P. Confluent monolayers of serum-starved HUVECs were treated for the times indicated with 10 nM thrombin or 0.5 μM SIP. Western blot analyses of active CRIB-bound Rac1 and Cdc42 are shown in the upper panels, and total proteins are shown in the lower panels. The results shown are representative of two independent experiments in which the effects of thrombin and S1P were directly compared. Fold stimulation of GTP-bound Rho proteins was determined following normalization against total GTPases.

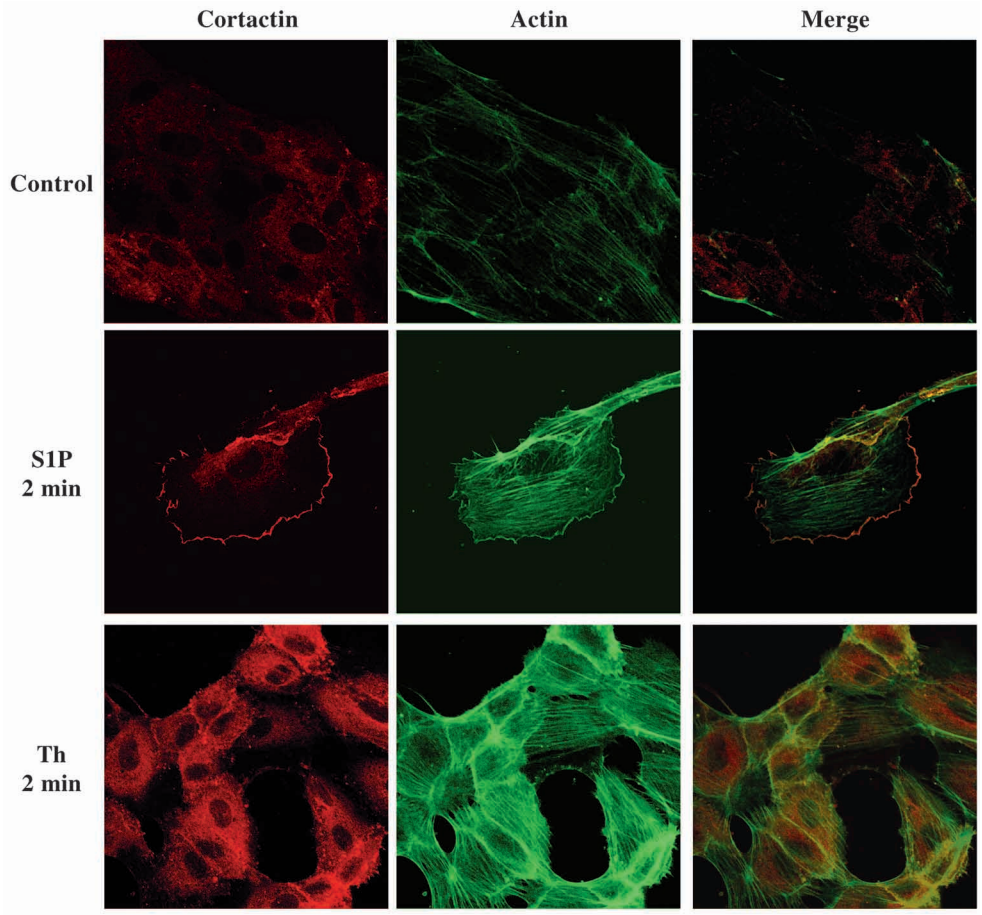

Fig. 3. S1P induces co-localization of cortactin and actin in lamellipodia. Serum-starved HUVECs were treated, or not (control), with S1P (0.5 μM) or thrombin (10 nM) for 2 minutes. After fixation and permeabilization, cells were stained with anti-cortactin antibody (left panels) and phalloidin-FITC (middle panels) and analyzed by confocal microscopy. Merged image pairs (right) highlight regions of co-localization (orange).

levels at 30 minutes. In contrast, S1P activates Rac1. Although stimulation of Rac1 by S1P is rather weak it is observed consistently. Stimulation can be detected as early as 2 minutes after S1P treatment (1.7-fold±0.3, *n*=3) and levels of GTP-bound Rac return to basal levels after 20 minutes.

Thus, major differences exist in the ability of thrombin and S1P to modulate the activity of Rho family proteins in HUVECs. First, the magnitude and time course of RhoA activation by these agonists is strikingly different. Thrombin is a considerably more potent activator of RhoA. Second, these agonists exert opposite effects on Rac1. Whereas S1P activates Rac1, thrombin inhibits Rac1 activity in resting cells.

Cortactin colocalizes with actin polymerization sites at the leading edge of S1P-treated cells

It has been reported that Rac1 is required for the translocation of cortactin from the cytoplasm to the cell periphery in Swiss 3T3 fibroblasts (Weed et al., 1998). Since we observed that S1P activates Rac1 in HUVECs, we examined whether S1P would induce peripheral relocalization of cortactin in these cells. To do so, cells were serum-starved, challenged with agonists and co-stained for cortactin and F-actin. As shown in Fig. 3, addition of 0.5 μM S1P induces the translocation of cortactin from the cytoplasm, where it resides in nontreated cells, to the perimeter of treated cells. Cortactin staining at the periphery is most intense from 1-5 minutes post-activation, thereafter a portion of the total cortactin pool returns to the cytoplasm (data not shown). This effect is independent of cell confluency since it occurs in both sparse (Fig. 3) and confluent cultures (Fig. 7 and data not shown). F-actin staining shows that S1P treatment causes a rapid extension of lamellipodia resulting in a notable increase in cell size. We observed a fine meshwork of actin filaments in the membrane protrusions bordered by a band of polymerized actin. Merger of fluorescent images reveals the co-localization (in orange) of cortactin and peripheral F-actin.

In sharp contrast, cells treated with thrombin do not spread. Rather, they become covered with longitudinal stress fibers that thicken at the lateral edges of cells. Cortactin staining in thrombin-treated cells (Fig. 7, lower panel) is excluded from the cell periphery and remains distributed throughout the cytoplasm with a minor enrichment in punctate structures at all time points examined (not shown).

Translocation of cortactin to the cell periphery requires Rac1 activation

To determine whether Rac activation participates in the control of cortactin translocation in HUVECs, cells were electroporated with an expression plasmid encoding a Myc-tagged dominant-negative Rac1 mutant (Rac1 N17). Electroporated cells were co-stained with the polyclonal anti-Myc and monoclonal anti-cortactin antibodies. As can be seen in Fig. 4 (top), staining of cortactin is excluded from the circumference of cells expressing Rac1 N17. Further, Rac1 N17 expression totally abolishes peripheral translocation of

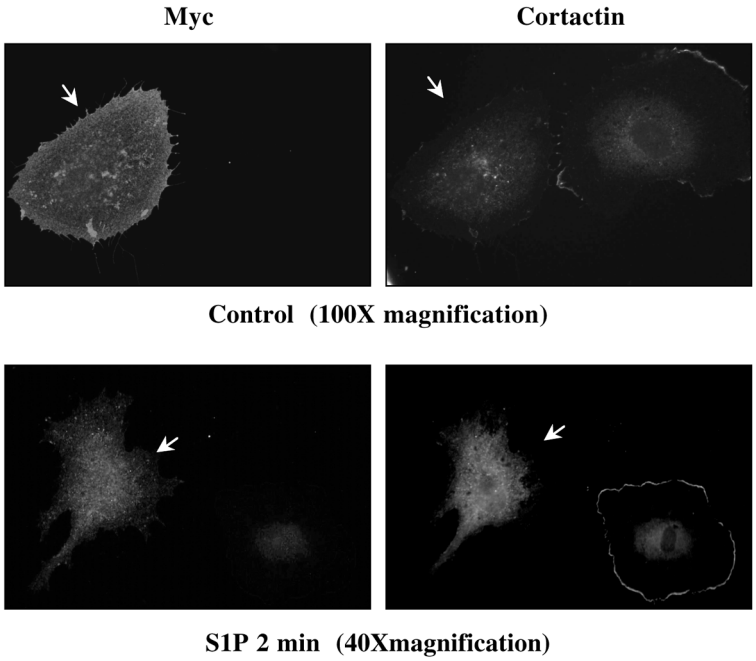

Fig. 4. Rac is involved in cortactin relocalization. HUVECs were electroporated with the pMT90-myc Rac1 N17 expression vector. Eighteen hours after electroporation, cells were detached, plated onto fibronectin-coated coverslips and allowed to spread for 8 hours before overnight serum starvation. Cells were then stimulated for 2 minutes with 0.5 μM S1P, fixed and stained for detection of myc expression (left panel) and cortactin localization (right panel). Arrows show myc-tagged RacN17-expressing cells.

cortactin in response to S1P (Fig. 4, bottom). It is noteworthy that in cells expressing this dominant-negative construct, S1P stimulates the formation of protrusive structures that extend in all directions. However, these extensions do not mature into smooth lamellipodia with F-actin-rich rims. We conclude from these results that S1P-induced stimulation of Rac1 is required for the translocation of cortactin from the cytoplasm to the edge of membrane extensions and for cortical actin polymerization.

Src activation is required for cortactin translocation to the cell periphery

Since cortactin is a well known substrate for $pp60^{c\text{-}Src}$, we examined the role of Src in S1P-induced cortactin translocation. First, Src kinase activity was determined following immunoprecipitation of Src family kinases from confluent monolayers of serum-starved HUVECs treated with thrombin or S1P. As shown in Fig. 5, S1P induces a rapid and transient increase in kinase activity. The maximal response (2.1-fold stimulation) is obtained 1 minute after S1P treatment, in accordance with the time of maximal cortactin recruitment to the cell periphery. In contrast, Src kinase activation by thrombin is delayed and weak in comparison with S1P. In both cases, kinase activity is tightly regulated and decreases to below basal levels after 15 minutes of agonist stimulation.

Although the overall Src kinase activation by S1P is modest, we did observe a localized activation of Src. Indeed, treatment of cells with S1P leads to the rapid appearance of active, phosphorylated Src, detected with anti-pSrc (pY^{418}), at the edge of lamellipodia (Fig. 6A). In fact, the immunofluorescence staining pattern obtained with the anti-p-Src (pY^{418}) antibody in S1P-treated cells was similar to that observed using the anti-cortactin antibody, except that staining of phospho-Src was considerably weaker and it was not excluded from nuclei. In contrast to S1P, we observed no peripheral staining of the active kinase in thrombin-treated cells. However, a slight enhancement of phospho-Src staining was detected in the nuclei of cells treated with either agonist. Thus, treatment of HUVEC with S1P triggers rapid Src activation at the edge of membrane ruffles, where cortactin staining is most intense. In agreement with this result, we also detected increased staining of tyrosine-phosphorylated proteins in S1P-stimulated cells. As can be seen in Fig. 6B, staining is most intense at the edge of membrane ruffles whereas in thrombin-stimulated cells phosphotyrosine-containing proteins are located in focal adhesions at the base of actin stress fibers.

In order to examine the role of Src in cortactin localization, serum-starved cells were pretreated with the pyrazolopyrimidine, PP2, a selective inhibitor of the Src family kinases (Hanke et al., 1996). As shown in Fig. 7, PP2 pretreatment at a concentration of 5 μM reduced the number and thickness of stress fibers, leaving only a fine belt of F-actin at cell perimeters. Under these conditions cells are less adherent to the substratum and cortactin staining is not modified. However, PP2 treatment totally precludes lamellipodia formation induced by S1P as well as peripheral staining of cortactin. In PP2-treated cells, we still observe the formation of stress fibers in response to the agonist addition, indicating that Src kinase activity is not required for

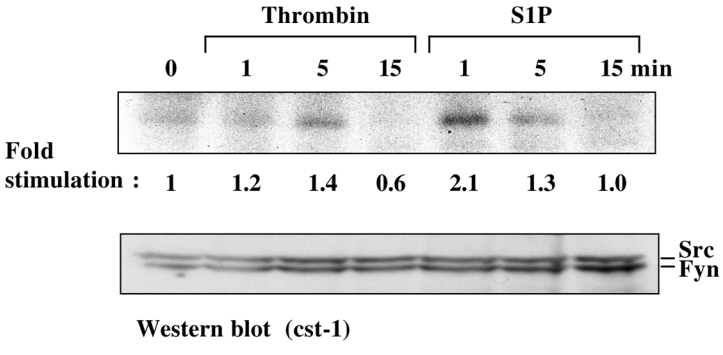

Fig. 5. S1P induces activation of Src kinase. Serum-starved confluent monolayers were treated with S1P (0.5 μM) or thrombin (10 nM) for the indicated times and an immunoprecipitation kinase assay was performed using anti-Src family kinase antibody (cst-1) as described in Materials and Methods. Src autophosphorylation is shown in the upper panel. Equal loading was verified by blotting aliquots of supernatants from each sample with cst-1.

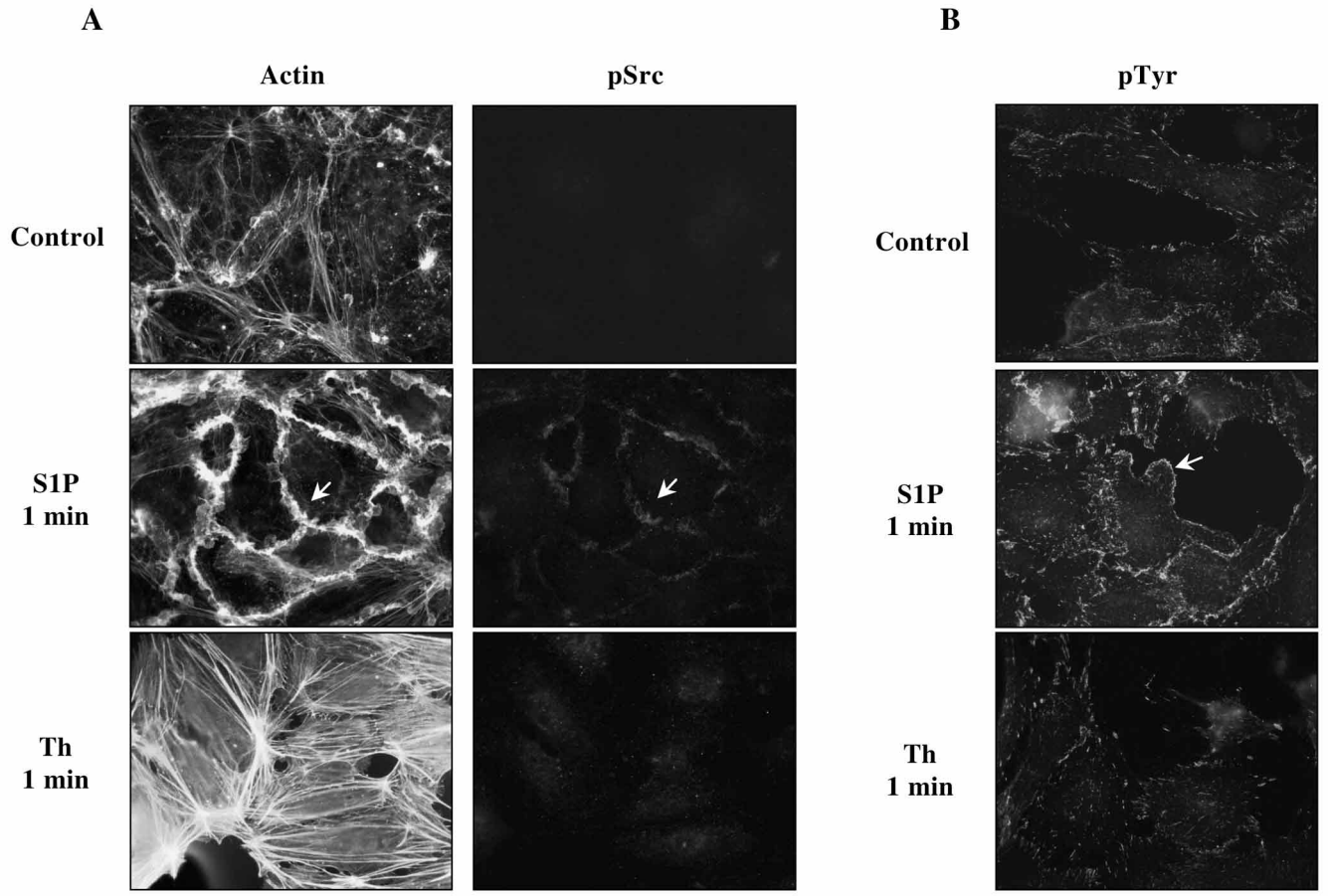

Fig. 6. S1P induces cortical targeting of pSrc. (A) Serum-starved HUVECs were treated for 1 minute with S1P (0.5 μM) or thrombin (10 nM), fixed and co-stained for active, phosphorylated Src (pSrc, right panels) and actin (left panels). The arrow in photos from S1P-treated cells depicts sites at the edge of membrane ruffles where pSrc appears (magnification, 1000×). (B) Cells treated with S1P (0.5 μM) or thrombin (10 nM) were fixed and stained with anti-phosphotyrosine antibody (pTyr). The arrow indicates enriched staining of tyrosine-phosphorylated residues in membrane ruffles.

actin filament bundling. Similar results were obtained using the parent compound PP1 (data not shown).

We next examined the contribution of Rho signaling to S1P-induced spreading and cortactin translocation. To do so, cells were pretreated with the compound Y-27632, an inhibitor of Rho kinase, a downstream target of Rho (Uehata et al., 1997). Similar to PP2 pretreatment, Y-27632 reduces stress fiber formation in nontreated cells without modifying the diffuse staining of cortactin. Interestingly, in cells challenged with S1P, Y-27632-pretreatment potently enhanced lamellipodia formation, as determined by F-actin staining. In addition, it potentiated the accumulation of cortactin in peripheral F-actin-rich ruffles. This Rho kinase inhibitor, which has previously been shown to attenuate thrombin-induced stress fiber formation (Carbajal et al., 2000), had no effect on the cytoplasmic staining of cortactin in thrombin-treated cells (not shown).

To determine whether Src activity is involved in activation of Rac by S1P, we performed Rac-GTP pull down assays on PP2-pretreated cells. The results shown in Fig. 8 (upper panel), revealed that Src inhibition does not reduce the level of active Rac1 following 2 or 10 minutes of stimulation with S1P. This observation indicates that Rac activation is not distal to Src kinase activation in these cells. In fact, we consistently observed a slight enhancement of both basal and agonist-stimulated Rac activity in the presence of PP2. Under these conditions, S1P-induced activation of Erk1/2 was not inhibited by PP2 either, indicating that Src is not upstream of Erk in these cells (Fig. 8, lower panel).

S1P-induced cell migration requires Src activation

S1P released from activated platelets is a potent chemoattractant that accounts for most of the chemotactic activity of serum for endothelial cells (English et al., 2000). We have seen above that Rac and Src cooperate to promote cortactin translocation and cell spreading. Therefore, we decided to further explore the role of Src involved in S1P-stimulated endothelial cell migration. To do so, we performed wounding assays on confluent monolayers of serum-starved HUVECs and observed cell migration towards the injured zone. Fixed cells were stained for F-actin and their morphology was examined by fluorescent microscopy. As shown in Fig. 9A, 2 hours after wounding few, if any, cells migrate in the absence of agonist. At this time the extension of small lamellipodia towards the open space can be observed. However, stimulation

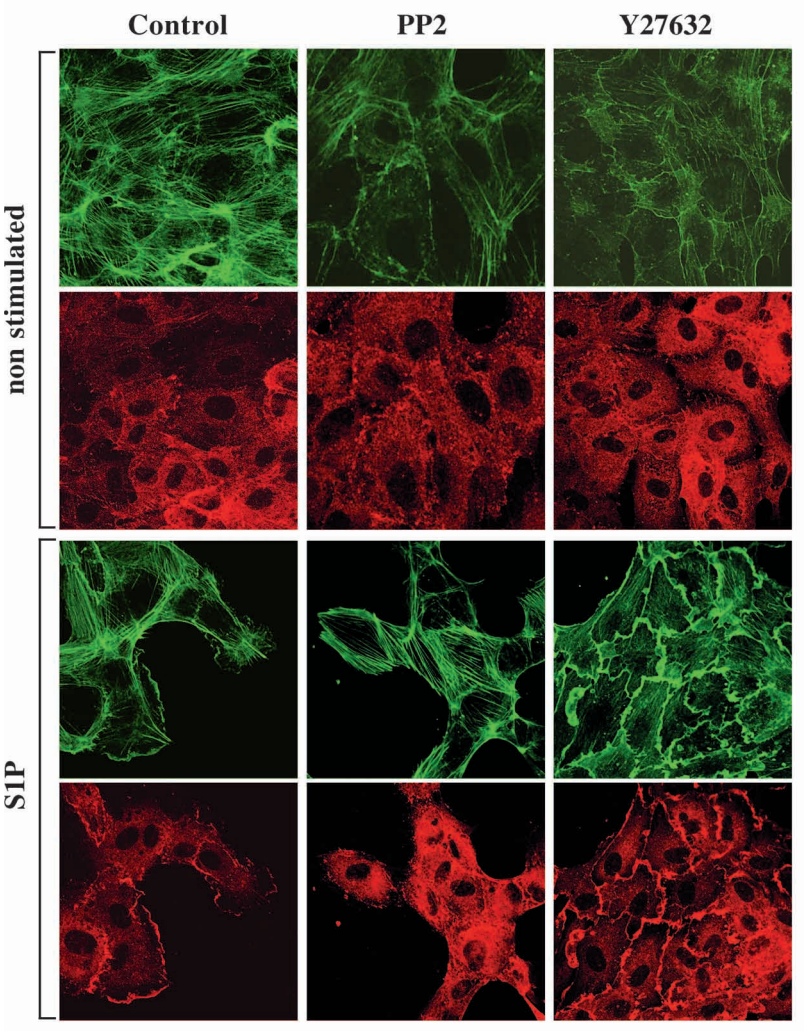

Fig. 7. Src inhibitor inhibits S1P-induced cortical localization of cortactin. Serum-starved HUVECs were pretreated with the Src kinase inhibitor, PP2 (5 μM), or Y27632 (7 μM), a Rho kinase inhibitor, for 30 minutes at 37°C; S1P was added for an additional 2 minutes before fixing and staining cells for cortactin (red) and actin (green). Confocal images of stained cells are shown.

of cells with S1P at the time of injury causes a marked increase in cell mobility characterized by an increase in F-actin staining and the extension of prominent membrane extensions in the direction of migration. Stress fibers in S1P-treated cells are mostly organized in parallel bundles oriented towards the damaged area. By 2 hours, the entire surface of the wound becomes covered with cells that have migrated towards the empty space. In contrast, thrombin treatment for 2 hours does not induce cell spreading or migration. Thrombin-stimulated cells become covered with thick, parallel actin cables anchored firmly to the substratum at vinculin-rich sites of attachment (not shown) and oriented in all directions. The majority of cell-cell contacts are lost and rather than spreading, cells remain contracted and devoid of protrusive membrane structures (i.e. lamellipodia or filipodia).

The role of Src in S1P-induced wound closure was examined in monolayers treated with PP2 prior to wounding. As shown in Fig. 9A, PP2 completely blocks the extension of membrane structures and cell migration in the presence of S1P suggesting that Src is required for movement of cells. It has previously been shown that cell migration depends on persistent activation of the MAP kinases, Erk 1/2, which regulate myosin motor function (Klemke et al., 1997). Consistent with this proposed role for Erk in cell migration, we observed a prolonged activation of Erk 1/2 by S1P in HUVECs that lasted for at least 240 minutes (Fig. 9B). Indeed, S1P-induced cell migration was inhibited by pretreatment with the MEK inhibitor U0126. As mentioned above, Src does not appear to act upstream of Erk1/2 in HUVECs since 5 μM PP2, a concentration that completely blocks cortactin translocation and cell migration has no effect on Erk1/2 activation (Fig. 8, lower panel). It is noteworthy for comparison with other studies that some Erk inhibition was observed at higher concentrations of PP2 (>50 μM, data not shown).

Discussion

By virtue of their direct effects on vascular endothelial cells, the soluble mediators thrombin and S1P represent an important link between the hemostatic system and blood vessel remodeling. Following vascular injury or during inflammation, thrombin generation is rapidly followed by platelet activation and release of S1P. The characterization of signaling mechanisms that control endothelial cell function by these ligands is essential for a complete understanding of angiogenesis and for the development of therapeutic approaches to regulate this process.

As can be seen in the present study, thrombin and S1P trigger distinct and opposite effects on the morphology of endothelial cells. Using-Rho directed toxins, mutant GTPases and pharmacological inhibitors, it has

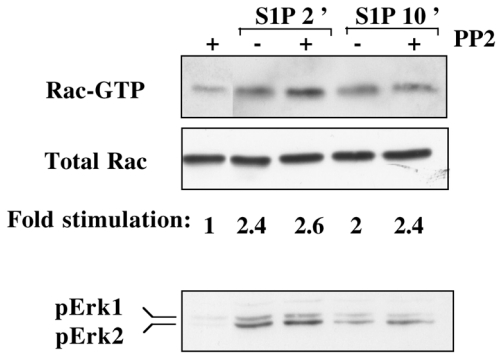

Fig. 8. Src kinase inhibition does not block Rac activation by S1P. Serum-starved HUVECs were pretreated with 5 μM PP2 for 20 minutes and 0.5 μM S1P was added for the indicated times. Active, CRIB-bound Rac1 is shown in the upper panel and the total amount of proteins in the middle panel. Fold stimulation of GTP-bound Rac was determined following normalization against total Rac. Blotting of the same samples with an anti-active-Erk1/2 antibody (pErk1/2) is shown below. The results shown are representative of three independent experiments.

previously been established that Rho family proteins participate in regulation of cytoskeletal responses to both ligands. Here we have extended our understanding of this regulation by defining the temporal and quantitative parameters of the agonist-induced changes in RhoA, Rac1 and Cdc42 activities. Our data demonstrate that thrombin is a potent activator of RhoA in endothelial cells compared with S1P. By contrast S1P activates Rac1 whereas thrombin inhibits this GTPase. In HUVECs, Cdc42 activity does not appear to be modulated by either agonist. These findings, together with previously published data, are consistent with a model in which retraction of endothelial cells by thrombin involves several coordinated events including: (1) rapid and robust stimulation of RhoA/Rho kinase-dependent acto-myosin contractility; (2) attenuation of Rac1 signaling; and (3) activation of additional signals, likely to involve phosphorylation events, that weaken adhesive cell-cell and cell-matrix interactions. We propose that the cytoskeletal response to S1P, which culminates in cell spreading and migration, is characterized by: (1) rapid and sustained activation of Rac1; (2) relatively weak and delayed activation of RhoA; and (3) Src kinase activation and membrane translocation of cortactin.

To our knowledge, this is the first determination of Cdc42 activity following thrombin stimulation of HUVECs. Intriguingly, activation of the thrombin receptor PAR-1 in platelets has been found to induce rapid and extensive activation of Cdc42 in a pull-down assay with the CRIB domain of PAK1 (Azim et al., 2000a). This difference highlights the importance of cellular context on the outcome of receptor signaling, as PAR-1 is coupled to a similar set of heterotrimeric G proteins in platelets and endothelial cells (i.e. G_i, G_q, $G_{12/13}$). Concerning RhoA, a similar profile of activation by thrombin in HUVECs has been reported (van Nieuw Amerongen et al., 2000). However, in this same study, these authors did not note the inhibition of Rac that we point out. It is possible that this difference may stem from differences in culture conditions that influence basal and stimulated Rac activity. Again, in sharp contrast to endothelial cells, Rac1 activity is increased by PAR-1 activation in platelets (Azim et al., 2000). Some clues may come from identification of the mechanisms underlying the negative regulation of Rac by thrombin in HUVECs. Interestingly, receptor-mediated inhibition of Rac activity was also recently observed in CHO cells stably expressing the EDG-5 receptor for S1P. In these cells, activation of EDG-5 leads to Rho activation and inhibition of both basal and IGF I-stimulated Rac activity as well as IGF-1-induced membrane ruffling and cell migration (Okamoto et al., 2000). The authors suggested that Rac inhibition via EDG-5 occurs by stimulation of a Rac-GTPase-activating protein. Further studies will be needed to identify the molecular events that lead to inhibition of Rac1 by thrombin and to determine its role in thrombin-induced morphological changes as well as its possible antagonistic effect on the stimulation of Rac-dependent events by various chemotactic agonists.

Indeed, we were surprised to find that thrombin inhibits rather than activates endogeneous Rac1 activity in primary cultured HUVECs, in light of our previous findings that overexpression of the dominant interfering Rac1 N17 mutant blocked the thrombin-induced cytoskeletal response (i.e. retraction and rounding) in a HUVEC-derived cell line, EaHy926 (Vouret-Craviari et al., 1998). The reason for this apparent discrepancy cannot be explained at present, yet it was recently reported that interference with the Rac pathway by recombinant adenovirus-based expression of either constitutively active or dominant interfering Rac1 mutants significantly increases permeability of unstimulated HUVEC monolayers (Wojciak-Stothard et al., 2001). In this study it was also shown that Rac1 inhibition weakens both adherens junctions and tight junctions. Thus, the inhibition of Rac1 that we observe in response to thrombin could serve to disrupt intercellular tethering forces and thereby facilitate cell rounding.

Concerning the effect of S1P on Rho GTPases in HUVECs, we observe an accumulation of active Rac1 and RhoA with no change in the level of active Cdc42. While our manuscript was in preparation, Paik et al. reported that 0.1 μM S1P strongly stimulates RhoA in HUVECs, using a similar GST-rhotekin

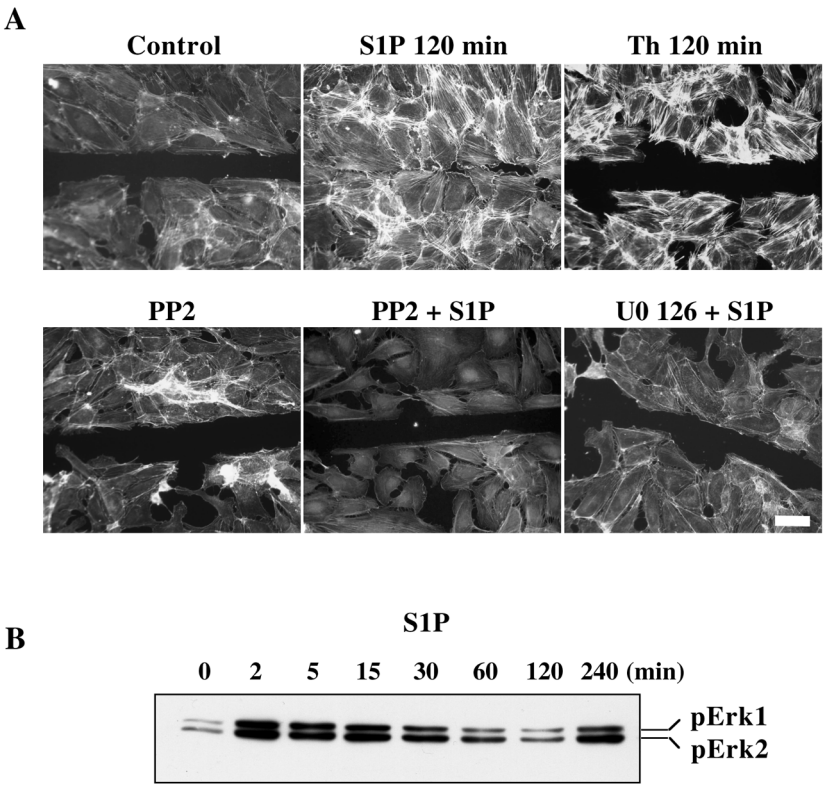

Fig. 9. S1P-induced HUVEC migration is inhibited by PP2. (A) Confluent monolayers of HUVECs were scraped with a 26G needle and the effect of 0.5 μM S1P or 10 nM thrombin on cell migration into the wounded area was observed in the presence or absence of 5 μM PP2 or 10 μM U0126. Two hours after agonist addition, cultures were fixed and the actin cytoskeleton was stained with phalloidin-FITC (bar, 40 μm). (B) Serum-starved HUVECs were treated with 0.5 μM S1P for the indicated times prior to analysis of Erk activation by western blotting using an anti-phospho Erk antibody (pErk1/2).

pull-down technique (Paik et al., 2001). However, by directly comparing the magnitude of the S1P effect with that of thrombin in these cells, our results reveal that activation of RhoA by S1P is in fact very weak. RhoA activation by S1P follows a similar time course to that of stress fiber accumulation; however, it is preceded by a rapid activation of Rac1 and extension of membrane ruffles. Interestingly, we see an enhancement of the S1P-stimulated membrane ruffles when the Rho effector, Rho kinase, is inhibited. We conclude from these results that the S1P-induced signaling that drives cell spreading, and peripheral actin polymerization is antagonized by the RhoA/Rho kinase pathway.

In addition to Rho GTPase activation, S1P triggers rapid translocation of the prominant Src substrat, cortactin, to peripheral membrane ruffles. Huang et al. have previously proposed a role for cortactin in endothelial cell migration based on their findings that overexpression of a cortactin mutant deficient in tyrosine phosphorylation impairs migration of ECV304 cells (Huang et al., 1998). Indeed, we show here that Src is rapidly activated by S1P in HUVECs. Kinase activation follows a similar time course to that of the S1P-induced appearance of active, phosphorylated Src in peripheral membrane ruffles. More importantly, we have found that Src activation is necessary for recruitment of cortactin to sites of actin polymerization at the edge of membrane ruffles and protrusion of lamellipodia. These findings suggest that cortactin plays an important role in linking Src kinase activation to cortical cytoskeleton reorganization. Consistent with this scheme, we were unable to detect thrombin-induced cortactin translocation.

In addition to the Src requirement for S1P-induced cortactin translocation, Rac was also found to be necessary for this effect since it was totally abolished following expression of Rac N17 in HUVECs. At present, it is not known how Rac1 activation controls cortactin translocation to the cell periphery. Parsons and colleagues were unable to detect a direct interaction between Rac and cortactin in fibroblasts (Weed et al., 1998), it is therefore probable that additional intermediates exist. Further, the signaling events that lead to Src and Rac1 activation by EDG receptors have not been defined.

A link between Src family kinases and Rac activation has been reported in the case of cytokine- or adhesive protein-dependent cell migration. In this context, the adaptor proteins p130Cas and Crk have been implicated in a signaling cascade leading to the activation of Rac through coupling to DOCK180 (Cheresh et al., 1999; Kiyokawa et al., 1998). Recently, Ohmori et al. have shown that S1P can stimulate the tyrosine phosphorylation of p130Cas and its interaction with Crk in HUVECs (Ohmori et al., 2001). Phosphorylation of p130Cas was proposed to be mediated by Fyn, rather than Src and, in light of the above mentioned role for p130Cas-Crk in migration, it was suggested that these events may be related to Rac. However, in our study we have demonstrated that Rac activation by S1P is not dependent on the stimulation of a Src family kinase sensitive to PP2, which suggests that an alternative mechanism exists.

Another interesting finding of the present study is that thrombin does not stimulate accumulation of active Src at the membrane, cortactin translocation nor cell migration towards an injury performed on a confluent monolayer. These results indicate that without the Rac1/cortactin/Src signaling system, the Rho/Rho kinase pathway is not sufficient for cell movement. Rather, thrombin appears to impede the basal movement of cells, consistent with a previously reported inhibitory effect of high-intensity Rho activation on cell migration (Nobes and Hall, 1999). Nonetheless, previous studies have indicated that thrombin can promote endothelial cell migration (Maragoudakis and Tsopanoglou, 2000). Our results do not contradict these findings, since these studies were performed either in vivo (Matrigel implants or chick chorioallantoic membranes) or in cultured cells exposed to thrombin for prolonged periods of time under conditions in which secondary effects of thrombin are not negligible. Indeed, it has been demonstrated that the pro-angiogenic effect of thrombin on endothelial cells results from the activation of multiple events including increased production and activation of matrix metalloproteinases, release of VEGF and upregulation of VEGF receptors (Duhamel-Clerin et al., 1997; Mohle et al., 1997; Tsopanoglou and Maragoudakis, 1999).

By performing systematic analyses of signaling pathways stimulated by thrombin and S1P in endothelial cells, we have gained new insight into the molecular mechanisms controlling cell shape changes and migration in response to specific agonists. We conclude that the Rac1/Src/cortactin signaling system represents a new component of agonist-stimulated signaling systems that regulate endothelial cell migration.

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