Thrombospondin Signaling of Focal Adhesion Disassembly Requires Activation of Phosphoinositide 3-Kinase*

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Thrombospondin is an extracellular matrix protein involved in modulating cell adhesion. Thrombospondin stimulates a rapid loss of focal adhesion plaques and reorganization of the actin cytoskeleton in cultured bovine aortic endothelial cells. The focal adhesion labilizing activity of thrombospondin is localized to the amino-terminal domain, specifically amino acids 17–35. Use of a synthetic peptide (hep I), containing amino acids 17–35 of thrombospondin, enables us to examine the signaling mechanisms specifically involved in thrombospondin-induced disassembly of focal adhesions. We tested the hypothesis that activation of phosphoinositide 3-kinase is a necessary step in the thrombospondin-induced signaling pathway regulating focal adhesion disassembly. Both wortmannin and LY294002, membrane permeable inhibitors of phosphoinositide 3-kinase activity, blocked hep I-induced disassembly of focal adhesions. Similarly, wortmannin inhibited hep I-mediated actin microfilament reorganization and the hep I-induced translocation of α-actinin from focal adhesion plaques. Hep I also stimulated phosphoinositide 3-kinase activity approximately 2–3-fold as measured in anti-phosphoinositide 3-kinase and anti-phosphotyrosine immunoprecipitates. Increased immunoreactivity for the 85-kDa regulatory subunit in anti-phosphotyrosine immunoprecipitates suggests that the p85/p110 form of PI 3-kinase was identified as a phosphatidylinositol kinase activity associated with TSP1-induced focal adhesions activity. Increased immunoreactivity for the 85-kDa regulatory subunit in anti-phosphotyrosine immunoprecipitates suggests that the p85/p110 form of phosphoinositide 3-kinase is involved in this pathway. In 32P-labeled cells, hep I increased levels of phosphatidylinositol (3,4,5)-triphosphate, the major product of phosphoinositide 3-kinase phosphorylation. These results suggest that thrombospondin signals the disassembly of focal adhesions and reorganization of the actin cytoskeleton by a pathway involving stimulation of phosphoinositide 3-kinase activity.

Thrombospondin (TSP) is an extracellular matrix protein involved in modulating cell adhesion, proliferation, and migration (for reviews on TSP, see Refs. 1 and 2). The structure of TSP1 consists of multiple domains which bind to various cell surface receptors and interact with other extracellular molecules. These receptors include heparan sulfate proteoglycans (3–5), sulfatides (6, 7), CD36 (8, 9), integrins (10), and the 52-kDa integrin-associated protein (11). Differential expression of these receptors and varying accessibility of the binding domains of TSP1 are potential mechanisms for regulating the effects of TSP1 on cell behavior. However, the multiplicity of domains and receptors that exist for TSP1 has made it difficult to determine the mechanisms by which TSP1 regulates cellular function.

This laboratory has examined the regulation of cell adhesion by TSP1. Bovine aortic endothelial (BAE) cells will attach to surfaces coated with TSP1, but spreading and cytoskeletal organization is not stimulated (10, 12). Soluble TSP1 induces a rapid disassembly of focal adhesions and reorganization of the actin cytoskeleton in spread endothelial cells (12). TSP1-induced disassembly of focal adhesions is accompanied by a dispersal of vinculin from the focal adhesion plaque, however, the αvβ3 integrin remains clustered (12, 13). The focal adhesion labilizing activity of TSP1 was localized to the amino-terminal heparin-binding domain, specifically amino acids 17–35 (14). Using a synthetic peptide, hep I, containing amino acids 17–35 of TSP1, we can specifically examine mechanisms involved in the disassembly of focal adhesions induced by TSP1 and assess the effect of this activity on endothelial cell function. Previously, we demonstrated that disassembly of focal adhesions in response to hep I is dependent upon cyclic GMP-dependent protein kinase (PKG) (15). Cells in which PKG is inhibited or deficient no longer respond to hep I. However, PKG does not appear to be activated by hep I and activation of PKG does not stimulate disassembly of focal adhesions. These results suggest that basal PKG activity is important for the process of focal adhesion disassembly stimulated by TSP1. The signaling mechanisms involved in regulating TSP1-induced disassembly of focal adhesions remain to be elucidated.

A candidate signaling cascade is that involving phosphoinositide 3-kinase (PI 3-kinase). This family of lipid kinases has been shown to be involved in mitogenesis, vesicle trafficking, integrin activation, and regulation of the actin cytoskeleton (for reviews on PI 3-kinase, see Refs. 16–18). PI 3-kinase was first identified as a phosphatidylinositol kinase activity associated with the virally encoded protein tyrosine kinase v-Src in cell extracts (19, 20). The structure of this form of PI 3-kinase was
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determined to be a heterodimer consisting of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit (16, 17). The p85/p110 PI 3-kinase is activated by interaction of the regulatory subunit, which contains two SH2 domains, one SH3 domain, and two proline-rich sequences, with receptor and non-receptor tyrosine kinases (16, 17). The 110-kDa subunit of PI 3-kinase, which has been shown to be inhibited directly by wortmannin and LY294002, catalyzes phosphorylation on the 3-position of phosphoinositides leading to increases in phosphatidylinositol (3,4)-bisphosphate (PtdIns (3,4)-P2) and phosphatidylinositol (3,4,5)-trisphosphate (PtdIns (3,4,5)-P3) (16, 21–25).

Although PI 3-kinase has been demonstrated to be involved in a multiplicity of cellular functions, the exact role of its lipid products in cell signaling is not clear. Recent evidence suggests that the 3-phosphorylated phosphoinositides initiate downstream signaling by rapidly recruiting and/or activating target proteins. For example, both PtdIns (3,4)-P2 and PtdIns (3,4,5)-P3 activate calcium-independent protein kinase C types ε, η, and δ (26, 27) and PtdIns (3,4,5)-P3 activates Akt kinase (28). PtdIns (3,4,5)-P3 has been demonstrated to compete with tyrosine-phosphorylated proteins for binding to SH2 domains, suggesting that PtdIns (3,4,5)-P3 may recruit SH2-containing proteins to specific compartments of the cell (29). Evidence is also accumulating that 3-phosphorylated phosphoinositides bind to and modulate actin-regulatory proteins such as profilin and gelsolin (30–32). The diversity of cellular events involving PI 3-kinases suggest that complex regulatory mechanisms must exist to generate specific signals.

Implication of PI 3-kinase in the regulation of the actin cytoskeleton prompted us to propose that PI 3-kinase is involved in the TSP1-induced disassembly of focal adhesions and reorganization of actin microfilaments. In current studies, we present evidence that TSP1 stimulates the activation of PI 3-kinase and that PI 3-kinase is a component of the signaling pathway mediating TSP1-induced disassembly of focal adhesions and reorganization of the actin cytoskeleton.

EXPERIMENTAL PROCEDURES

Materials—Hep I (ELTGAARKGSGRRLVKGPD) and scrambled hep I (RSIKAGTGLERDLKPGARVG) were synthesized, purified, and analyzed by the UAB Comprehensive Cancer Center/Peptide Synthesis and Analysis shared facility. Phosphatidylinositol was purchased from Avanti Polar Lipids (Alabaster, AL). Wortmannin, mouse monocular antibody to α-actinin (clone BM752), protein A-Sepharose, crude brain phosphoinositides, and phosphatidylinositol 4-phosphate were purchased from Sigma. Phosphatidylinositol 4,5-bisphosphate (PtdIns (4,5)-P2) was obtained from American Radiolabeled Chemicals (St. Louis, MO), and LY294002 from Biomol (Plymouth Meeting, PA). Anti-p85 PI 3-kinase (rabbit polyclonal) and anti-phosphotyrosine (mouse monoclonal) antibodies were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). [32P]ATP was obtained from ICN. [32P]ATP was obtained from [32P]ATP sample and 17.5 mM MgCl2. The samples were vortexed and incubated for 10 min at 37 °C. The reaction was started by adding 20 μl of kinase buffer containing 17.5 μM ATP (25 μl of [32P]ATP/sample) and 17.5 mM MgCl2. The samples were vortexed and incubated for 10 min at 37 °C. Reactions were stopped by adding 500 μl of chloroform/methanol/water (3:6:1). Lipids were then extracted by adding 175 μl of chloroform and 175 μl of 1 M HCl, vortexing, and centrifuging to separate the phases. The lower phase was removed, added to 630 μl of methanol, 1 M HCl/chloroform (16:15:8:1), vortexed, and centrifuged. The lower phase was removed and the lipids separated by TLC on Silica Gel 60 plates precoated with 1% potassium oxalate. The plates were developed in methanol/chloroform/water/ammonium hydroxide (8:6:2:1) when phosphatidylinositol was the substrate and chloroform/acetone/methanol/acetatic acid/water (40:15:13:12:7) when PtdIns (4,5)-P2 was the substrate (37). The TLC plates were exposed for autoradiography and quantitated using video-enhanced densitometry. The positions of PtdIns-P, PtdIns-P2, and PtdIns (4,5)-P3 as migrated on the TLC plate were visualized by exposing the plate to I, vapor and identified using purified standards.

To prepare the lipid substrates, phosphatidylinositol was dried from the purchased chloroform solution and then sonicated into kinase buffer containing 0.2% deoxycholate and 3.5 mM dithiothreitol until clear to achieve a concentration of 3 μg/ml. PtdIns (4,5)-P2 was prepared for phosphorylation by drying down with an equal amount of phosphatidylinositol and sonicating into kinase buffer containing 3.5 mM dithiothreitol to get concentrations of 1.5 μg/ml PtdIns (4,5)-P2 and 1.5 μg/ml phosphatidylinerine.

23P Labeling of Intact Cells and Examination of 23P-Labeled Lipids—Analysis of hep I-stimulated incorporation of 23P into the lipids in intact cells was performed as described (38). BAEC cells were grown to approximately 80% confluency in a 100-mm culture dish. Cells were incubated overnight in 0.2% FBS/DMEM. The media was changed to phosphate-free DMEM and incubated for 1 h. Cells were then incubated for 30 min containing 1 μM hep I. The cells were stimulated for 10 min. The media was aspirated and the cells scraped and collected in 750 μl of methanol, 1 M HCl (1:1); 20 μg of crude phosphoinositides was added as a carrier. The lipids were extracted by adding 380 μl of chloroform and shaking 15 min at room temperature. Samples were centrifuged to separate the phases and the upper phase discarded. The extracted lipids were washed twice with 500 μl of methanol, 0.1 M EDTA (1:0.9), separated by TLC, and analyzed as described above. These procedures were developed in chloroform/acetone/methanol/acetatic acid/water (40:15:13:12:7) (37).

Immunofluorescence—BAEC cells were stained with Biodipy-phallidin or α-actinin as described previously (14). Cells stained for α-actinin were extracted with Triton X-100 buffer (20 mM Tris, pH 7.4, 50 mM NaCl, 1 mM EGTA, 5 mM EDTA, 50 mM sodium pyrophosphate, 50 μM sodium fluoride, 100 μM sodium orthovanadate, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 0.5% Triton X-100) for 5 min on ice prior to fixation.

Immunoblotting—Soluble proteins were extracted in Triton X-100 buffer as described above. The extracted cells were rinsed 2 times with ice-cold phosphate-buffered saline on ice and the insoluble proteins collected in electrophoresis sample buffer. Protein concentrations were determined by the method of Lowry (39) after acid precipitation. Samples (30 μg) were resolved by electrophoresis on SDS-polyacrylamide gels and transferred to nitrocellulose. Blots were blocked, probed, and developed by enhanced chemiluminescence (NEN Life Science Products, Boston, MA).
RESULTS

Inhibitors of PI 3-Kinase Block Focal Adhesion Disassembly Induced by the Active Sequence of TSP1—A statistically significant decrease in focal adhesions can be observed in spread BAE cells 8 min following administration of hep I, the active sequence from TSP1, with disassembly complete after 20 min and stable for at least 60 min (Fig. 1). To determine whether PI 3-kinase was involved in TSP1-induced disassembly of focal adhesions, cells were treated with hep I in the presence of wortmannin or LY294002, membrane permeable inhibitors of PI 3-kinase activity. Wortmannin, an irreversible inhibitor acting at the lipid-binding site (21, 22, 24), completely blocked the hep I-stimulated disassembly of focal adhesions at concentrations as low as 2.5 nM (Fig. 2A). LY294002, a reversible inhibitor which competes for the ATP-binding site (21, 23), inhibited hep I activity at concentrations as low as 5 μM (Fig. 2B). Published data shows that at these low concentrations, wortmannin and LY294002 do not directly inhibit other lipid kinases or phospholipase A2 (21–24, 77). Focal adhesion disassembly stimulated by the intact TSP1 protein was also completely inhibited by wortmannin and LY294002 (Table I). As previously reported (14), a synthetic peptide containing the scrambled amino acid sequence of hep I did not result in the disassembly of focal adhesions (Table I).

Wortmannin Inhibits Hep I-mediated Actin Reorganization—Previous work demonstrated that TSP1 and hep I stimulate reorganization of the actin cytoskeleton (12, 14). Control cells display thick stress fibers terminating at the focal adhesion plaques, while the microfilaments in hep I-treated cells appeared thinner, with a more peripheral distribution (12, 14). To determine if PI 3-kinase was involved in the TSP1-induced rearrangement of the actin cytoskeleton, the microfilaments were examined in control and hep I-treated BAE cells pretreated in the absence or presence of 5 nM wortmannin. Consistent with previous observations, phallicidin-stained F-actin microfilaments in hep I-treated cells (Fig. 3b) exhibit a more peripheral distribution as compared with the more centrally located stress fibers terminating at focal adhesion plaques in control cells (Fig. 3a). The hep I-stimulated reorganization of the actin cytoskeleton was completely inhibited by 5 nM wortmannin (Fig. 3d) with F-actin staining similar to the non-hep I-treated controls. Treatment with wortmannin alone did not appear to affect F-actin microfilament organization (Fig. 3c).

These results suggest that PI 3-kinase activity is required for the rearrangement of actin microfilaments induced by hep I.

α-Actinin is an actin-regulatory protein which binds directly to actin filaments as well as to other components of focal adhesions forming the protein network anchoring microfilaments to the membrane at contact sites (40, 41). The localization of α-actinin was examined in control and hep I-treated cells in the absence or presence of 5 nM wortmannin. Since previous studies have reported that antibody accessibility to focal adhesion localized α-actinin is limited (42), cells were extracted with Triton X-100 buffer prior to fixation and staining. Under these conditions, antibody specific for α-actinin intensely stained focal adhesion plaques (Fig. 4a). Staining for

**FIG. 1.** Time course of hep I-stimulated disassembly of focal adhesions. BAE cells were grown on glass coverslips until near confluence, rinsed, and then incubated with DMEM for 1 h, treated with 1 μM hep I for various times, fixed with 3% glutaraldehyde, and examined for the presence of focal adhesion positive cells by interference reflection microscopy as described under “Experimental Procedures.” n = 3, error bars represent S.D.

**FIG. 2.** Wortmannin and LY294002 inhibit the hep I-induced disassembly of focal adhesions. BAE cells were grown on glass coverslips until near confluence, rinsed, and then incubated with DMEM for 1 h, preincubated with various concentrations of wortmannin (A) or LY294002 (B) for 5–10 min, treated with DMEM (control) or 1 μM hep I for 30 min, fixed with 3% glutaraldehyde, and examined for the presence of focal adhesion positive cells by interference reflection microscopy. n = 1–8; error bars represent S.D.
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TABLE I
Inhibition of TSP1-induced disassembly of focal adhesions

| Treatment*          | Cells positive for focal adhesions |
|---------------------|-----------------------------------|
| Control             | 56                                 |
| Hep I               | 28                                 |
| Scrambled hep I     | 50                                 |
| TSP1                | 34                                 |
| TSP1 + wortmannin   | 51                                 |
| TSP1 + LY294002     | 47                                 |

* BAE cells were grown on glass coverslips until near confluence, rinsed and then incubated with DMEM for 1 h, preincubated with 5 nM wortmannin or 5 μM LY294002 for 5 min, treated with DMEM (control), 0.1 μM hep I, 0.1 μM scrambled hep I, or 10 μg/ml TSP1 for 50 min, fixed with 3% glutaraldehyde, and examined for the presence of focal adhesion positive cells by interference reflection microscopy. n = 2.

α-actinin was also observed along actin microfilaments in a beaded pattern. In hep I-treated cells, a decrease in α-actinin staining of focal adhesions in comparison to controls was observed (Fig. 4b), although the beaded pattern of staining was only slightly decreased. Wortmannin inhibited the hep I-induced decrease in α-actinin staining of focal adhesion plaques (Fig. 4d) without affecting α-actinin localization in control cells (Fig. 4c). These results suggest that PI 3-kinase activity is also required for the hep I-stimulated redistribution of α-actinin from focal adhesions.

To confirm the relocalization of α-actinin in response to hep I stimulation, immunoblotting was used to examine the distribution of α-actinin in the Triton X-100 soluble and insoluble fractions from control and hep I-treated cells. Under basal conditions, α-actinin was detected in the insoluble cytoskeletal fraction almost exclusively. However, in hep I-treated cells we observed a time-dependent increase in α-actinin in the soluble fraction (Fig. 5). These data suggest that α-actinin translocates to the detergent-soluble fraction as part of the process of hep I-mediated focal adhesion dissolution.

Hep I Stimulates PI 3-Kinase Activity—To further examine the role of PI 3-kinase in the TSP1-induced disassembly of focal adhesions, we measured the activation of PI 3-kinase in response to hep I. Complete inhibition of hep I activity by low nanomolar concentrations of wortmannin suggested that heterodimeric forms of PI 3-kinase, containing an 85-kDa regulatory subunit, mediate the disassembly of focal adhesions by hep I (21, 43, 45). Other isoforms of PI 3-kinase that lack the 85-kDa regulatory subunit require higher concentrations of wortmannin for inhibition (21, 43–45). Previously, researchers have demonstrated that PI 3-kinase in immunoprecipitates from agonist-stimulated cells show an enhanced activity when assessed in vitro kinase assays (16, 17). Therefore soluble lysates from control and hep I-treated cells were immunoprecipitated with antibody recognizing the 85-kDa regulatory subunit of PI 3-kinase. Similar to published studies, the immunoprecipitates were assayed for PI 3-kinase activity using phosphatidylinositol as a substrate (16, 17). Hep I stimulated the activity of PI 3-kinase 2.1-fold in the anti-p85-PI 3-kinase immunoprecipitates (Fig. 6). Insulin, which has been demonstrated to activate PI 3-kinase in various cell types (46–49), stimulated the activity of PI 3-kinase 4-fold in this assay. These data demonstrate that PI 3-kinase is activated in response to hep I addition.

Agonist-stimulated activation of PI 3-kinase often occurs by a pathway involving activation of tyrosine kinases and is frequently measured in anti-phosphotyrosine immunoprecipitates (16, 17). Therefore anti-phosphotyrosine (PY20) immunoprecipitates from control and hep I-treated cells were assayed for PI 3-kinase activity. Using phosphatidylinositol as a substrate, a 2.3-fold increase in PI 3-kinase activity was detected in the anti-phosphotyrosine immunoprecipitates from hep I-treated cells (data not shown), similar to what was observed in the anti-p85-PI 3-kinase immunoprecipitates. However, a considerable level of basal phosphatidylinositol kinase activity was also observed. To assay specifically for phosphorylation by receptor-stimulated PI 3-kinase, these assays were performed using PtdIns (4,5)-P₃ as a substrate. Using PtdIns (4,5)-P₃ as a substrate, hep I stimulated the activation of PI 3-kinase in the anti-phosphotyrosine immunoprecipitates 2.9-fold (Fig. 7, A and B). Similar activation of PI 3-kinase was also observed in cells treated with 10 μg/ml intact TSP1 (Fig. 8). Scrambled hep I peptide which does not induce focal adhesion disassembly did not stimulate PI 3-kinase activity (Fig. 8).

The activation of PI 3-kinase by hep I was observed as early as 2 min and maintained for the entire 120-min time course. The time course of PI 3-kinase activation by hep I was nearly identical to the time course of focal adhesion disassembly induced by hep I (compare Fig. 1 with Fig. 7B). Hep I-induced (10 min) PI 3-kinase activity was completely inhibited by the in vitro addition of 50 nM wortmannin, further demonstrating the specificity of the assay (Fig. 7A). To confirm that p85/p110 PI 3-kinase was involved in the increased PI 3-kinase activity in the anti-phosphotyrosine immunoprecipitates, immunoblotting with antibodies specific for the 85-kDa regulatory subunit was performed. A time-dependent increase in p85 immunoreactivity was observed in the anti-phosphotyrosine immunoprecipitates following treatment with hep I (Fig. 7B, inset).

To determine if hep I or TSP1 stimulate an increase in the tyrosine phosphorylation of the 85-kDa regulatory subunit, anti-p85-PI 3-kinase immunoprecipitates were immunoblotted with anti-phosphotyrosine antibodies. Tyrosine phosphorylation of the 85-kDa regulatory subunit was not observed in control or treated samples (Fig. 9). However, a tyrosine phosphorylated protein of ~78 kDa was observed and had increased immunoreactivity in samples from hep I and TSP1-treated cells compared with controls. Antibodies specific for p85-PI 3-kinase did not recognize the ~78-kDa band (Fig. 9).

Hep I Stimulates PtdIns (3,4,5)-P₃ Production in Intact Cells—The primary in vivo products of p85/p110 PI 3-kinase phosphorylation are PtdIns (3,4)-P₂ and PtdIns (3,4,5)-P₃ (25). To further establish that TSP1 activates this type of PI 3-kinase, the levels of PtdIns (3,4,5)-P₃ were examined in cells stimulated with hep I. To our knowledge, the only in vivo mechanism for generating PtdIns (3,4,5)-P₃ is the phosphorylation of PtdIns (4,5)-P₂ by PI 3-kinase. The production of PtdIns (3,4)-P₂ can occur by mechanisms involving phosphorylation and dephosphorylation (25). Therefore, measurement of PtdIns (3,4,5)-P₃ levels provides clear evidence for the activation of PI 3-kinase by an agonist. BAE cells were labeled with ³²Pᵣ, treated with hep I (10 μM) for 10 min, and the lipids extracted and separated by TLC. BAE cells were also stimulated with insulin (10 μg/ml) as a positive control. Hep I stimulated a 4.1-fold increase in PtdIns (3,4,5)-P₃ levels over non-treated controls (Fig. 10). Insulin stimulated a 3.9-fold increase in PtdIns (3,4,5)-P₃. These results confirm the in vitro observation that the activation of PI 3-kinase is stimulated by hep I. Together with the inhibitor data, these two observations support a role for a tyrosine kinase regulated PI 3-kinase in the TSP1 signaling pathway leading to focal adhesion disassembly.

DISCUSSION

Cell adhesion involves receptor-mediated cell surface interactions with the extracellular matrix (40, 41, 50, 51). These interactions play a central role in the organization of the cytoskeleton, thereby regulating cell shape and function. Focal adhesions are specialized structures linking the extracellular matrix to the actin microfilaments through integrin and syn-
The structure of the focal adhesion plaque consists of an elaborate network of interconnecting proteins anchoring the microfilaments to the membrane at the contact site. Focal adhesions are dynamic and highly regulated structures which assemble and disassemble during normal cellular functioning (40, 41, 50, 51). During the formation of focal adhesions, structural components are recruited and assembled to anchor the microfilaments to the adhesion site. Several important groups of proteins have been identified in the assembly process such as protein kinases, lipid kinases, and small GTP-binding proteins (52–57). Although, roles for protein kinase C and cyclic AMP-dependent protein kinase have been identified in disassembly of focal adhesions (58–65), our understanding of the disassembly process is limited.

Previously, we demonstrated that TSP1-induced disassembly of focal adhesions in endothelial and smooth muscle cells is dependent on PKG (15). However, TSP1 does not stimulate the activation of PKG. These results indicated that basal PKG activity is important for the process of focal adhesion disassembly. Moreover, previous studies suggest that the active sequence of TSP1 interacts with a protein receptor at the cell surface (a receptor candidate has recently been identified). In this paper, we show that activation of PI 3-kinase is essential for TSP1 signaling of focal adhesion disassembly and reorganization of the actin cytoskeleton. This conclusion is based on the following data: 1) two structurally different inhibitors of PI 3-kinase activity, wortmannin and LY294002, completely blocked the hep I-induced disassembly of focal adhesions; 2) wortmannin inhibited the hep I-induced reorganization of the actin cytoskeleton; 3) hep I stimulated activation of PI 3-kinase with kinetics nearly identical to that of focal adhesion disassembly; and 4) hep I stimulated an increase in the cellular levels of PtdIns (3,4,5)-P3, the major product of PI 3-kinase phosphorylation. These results are the first direct evidence that TSP1/hep I initiates intracellular signaling. The relationship between the activation of PI 3-kinase and basal PKG activity during TSP1 signaling remains to be determined.

Since the initial discovery of PI 3-kinase, several different isoforms have been identified which have differential sensitivities to wortmannin inhibition (21, 43–45, 69–72). The observation that low concentrations of wortmannin (2.5 nM) inhibited the TSP1-induced disassembly of focal adhesions suggests that a p85/p110 PI 3-kinase mediated this response. In addition, hep I stimulation of PI 3-kinase was detected by immunoprecipitation with antibody recognizing the 85-kDa regulatory subunit. Hep I also induced an increase in immunoreactivity for the 85-kDa regulatory subunit in anti-phosphotyrosine immunoprecipitates correlating to the increase in PI 3-kinase activity. These observations are consistent with the involvement of the p85/p110 form of PI 3-kinase. Furthermore, p85/p110 PI 3-kinase has been localized to focal adhesions in endothelial cells and fibroblasts suggesting a role for this enzyme in focal adhesion regulation (41, 68). However, these

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results do not exclude the possibility that other forms of PI 3-kinase may also play a role in TSP1 signaling. Activation of p85/p110 PI 3-kinase has been demonstrated to occur through the interaction of the 85-kDa regulatory subunit with other proteins, often tyrosine-phosphorylated proteins. Detection of TSP1-induced PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates suggests that the extent of tyrosine phosphorylation of PI 3-kinase or a tightly associated protein was increased in hep I-treated cells. Tyrosine phosphorylation of the 85-kDa subunit has been reported in response to certain agonists (16, 17), however, the relationship between tyrosine phosphorylation of the regulatory subunit and catalytic activity is unclear. We were unable to detect tyrosine phosphorylation of PI 3-kinase, suggesting that immunoprecipitation of PI 3-kinase activity with anti-phosphotyrosine antibodies most likely occurred as a result of the association of PI 3-kinase with another protein which was tyrosine phosphorylated. Consistent with this conjecture, a tyrosine-phosphorylated protein migrating at 78 kDa was detected in anti-p85-PI 3-kinase immunoprecipitates. Current studies are di-

**Fig. 4. Wortmannin inhibits hep I-mediated redistribution of α-actinin.** BAE cells were grown on glass coverslips until near confluence, incubated overnight in 0.2% FBS/DMEM, preincubated with 5 nM wortmannin for 5 min (c and d), treated with DMEM (a and c) or 10 μM hep I (b and d) for 30 min, extracted with Triton X-100 buffer for 5 min on ice, and fixed with 3% formaldehyde. α-Actinin was localized by indirect immunofluorescence using a monoclonal antibody specific for α-actinin and examined by epifluorescence microscopy. Bar = 10 μm.

**Fig. 5. Hep I stimulates an increase in soluble α-actinin.** BAE cells were grown on glass coverslips until near confluence, rinsed, and then incubated with DMEM for 1 h, treated with 10 μM hep I for various times, and extracted with Triton X-100 buffer for 5 min on ice. The soluble fraction (30 μg/lane) was resolved by electrophoresis on a 7.5% polyacrylamide gel and transferred to a nitrocellulose membrane. Equal protein loading was confirmed by Ponceau S staining of the membrane prior to immunoblotting with a monoclonal antibody specific for α-actinin (1:100,000). Results are representative of four separate experiments.

**Fig. 6. Hep I stimulates PI 3-kinase activity in anti-p85-PI 3-kinase immunoprecipitates.** Shown is an autoradiograph of PI 3-kinase activity in anti-p85-PI 3-kinase (Transduction Laboratories) immunoprecipitates. BAE cells were preincubated overnight in 0.2% FBS/DMEM, treated with DMEM, 10 μM hep I, or 10 μg/ml insulin for 10 min, and soluble lysates obtained for immunoprecipitation as described under “Experimental Procedures.” PI 3-kinase activity was assayed using phosphatidylinositol as a substrate. The position PtdIns-P migrated on the TLC plate was visualized by exposing the plate to I2 vapor and identified using purified standards. In comparison with DMEM treated controls, hep I stimulated a 2.1-fold increase in PI 3-kinase activity in the anti-p85-PI 3-kinase immunoprecipitates (n = 3).
rected at determining the identity of this protein and its relationship to PI 3-kinase in the TSP1 signaling of focal adhesion disassembly.

The major products of receptor-stimulated PI 3-kinase activation, PtdIns(3,4)-P2 and PtdIns(3,4,5)-P3, have recently become recognized as a second messenger involved in transducing extracellular signals regulating the actin cytoskeleton (16, 17), however, the mechanism by which these phosphoinositides carry out this function is unclear. Recent reports have shown that certain actin-regulating proteins which bind

**FIG. 7.** Hep I stimulates PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates. Shown is an autoradiograph of PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates. BAE cells were preincubated overnight in 0.2% FBS/DMEM, stimulated with 10 μM hep I for various times, and soluble lysates obtained for immunoprecipitation as described under “Experimental Procedures.” PI 3-kinase activity was assayed using PtdIns(4,5)-P2 (A) as a substrate. The position PtdIns(4,5)-P2, migrated on the TLC plate was visualized by exposing the plate to I2 vapor and identified using purified standards. Quantitation of PI 3-kinase activity in A is shown in B (n = 1–4; error bars represent S.E.). Inset, protein from the anti-phosphotyrosine immunoprecipitates was immunoblotted with a monoclonal antibody specific for p85-PI 3-kinase.

**FIG. 8.** TSP1 stimulates PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates. Autoradiograph of TSP1-stimulated PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates compared with controls and hep I. BAE cells were preincubated overnight in 0.2% FBS/DMEM, stimulated with 0.1 μM hep I, or 10 μg/ml TSP1 for 10 min, and soluble lysates obtained for immunoprecipitation as described under “Experimental Procedures.” PI 3-kinase activity was assayed using PtdIns(4,5)-P2 as a substrate. The position PtdIns(4,5)-P2 migrated on the TLC plate was visualized by exposing the plate to I2 vapor and identified using purified standards.

**FIG. 9.** Hep I and TSP1 do not stimulate tyrosine phosphorylation of p85-PI 3-kinase. Shown is an immunoblot of anti-p85-PI 3-kinase monoclonal antibodies (RC20, 1:2500). The same blot was stripped and then reprobed with anti-p85-PI 3-kinase monoclonal antibodies (1:1000). BAE cells were preincubated overnight in 0.2% FBS/DMEM, stimulated with 0.1 μM hep I, 0.1 μM scrambled hep I, or 10 μg/ml TSP1 for 10 min, and soluble lysates obtained for immunoprecipitation.

**FIG. 10.** Hep I stimulates an increase in PtdIns(3,4,5)-P3 levels. Shown is an autoradiograph of PtdIns(3,4,5)-P3 levels. BAE cells (100-mm dish) were incubated overnight in 0.2% FBS/DMEM, followed by incubation for 60 min in phosphate-free DMEM, and then labeled for 60 min with 1.6 mCi/ml 32P. Cells were then treated with DMEM, 10 μM hep I, or 10 μg/ml insulin for 10 min, and lipids extracted with chloroform. The positions PtdIns-P3 migrated on the TLC plate was visualized by exposing the plate to I2 vapor and identified using purified standards. Results are representative of two separate experiments.

PtdIns (4,5)-P2 have equal or greater affinity for PtdIns (3,4,5)-P3 (37, 73, 74). PtdIns (4,5)-P2 also binds α-actinin, enhancing its ability to promote actin polymerization. It has been suggested that α-actinin may be important for maintaining or stabilizing microfilament attachment in mature focal adhesions (40). Our data show that α-actinin is redistributed from focal adhesion plaques in response to hep I. Thus, one possibility is that α-actinin is a target for the second messenger action of PtdIns (3,4,5)-P3 involved in the mechanism of TSP1-induced disassembly of focal adhesions.

The carboxyl-terminal domain of TSP1 binds to the integrin-associated protein and enhances spreading of C32 human melanoma cells on vitronectin (67). The enhanced spreading induced by the carboxyl-terminal domain was blocked by 10 nM wortmannin (67), suggesting that at least one other active domain of TSP1 signals through the activation of PI 3-kinase. In fact, there are numerous reports describing a role for PI 3-kinase in agonist-induced activation of specific integrins leading to increased attachment (18). These studies appear contrary to this report suggesting a role for PI 3-kinase in the disassembly of focal adhesions. There are several possible ex-
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First, integrin-mediated attachment and the formation of focal adhesions and stress fibers are two distinct and temporally separated stages of adhesion (33, 34). In fact, Nobes et al. (35) have demonstrated that PI 3-kinase activity is not required for the formation of focal adhesions and stress fibers induced by lysophosphatidic acid, bombesin, or microinjected rho protein. Furthermore, integrin activation/reorganization does not appear to be involved in the TSP1-mediated disassembly of focal adhesions, since αbβ3 integrin remains clustered (12, 13). Second, our experiments were performed on adherent cells containing focal adhesions and stress fibers, rather than with non-adherent cells lacking these structures. Localization and availability of specific signaling components can differ following adhesion (51). Finally, the work implicating PI 3-kinase in integrin activation has been performed mostly on immune and hemopoietic cells; different cell types often respond in different ways.

Focal adhesion kinase (FAK) has been demonstrated to associate with and activate PI 3-kinase in vitro and in lysates from stimulated cells (75, 76). To determine if TSP1 stimulates the association of PI 3-kinase with FAK, anti-FAK immunoprecipitates were measured for PI 3-kinase activity. Preliminary results suggested that hep I stimulated a slight increase in PI 3-kinase activity in anti-FAK immunoprecipitates (data not shown). However, the PI 3-kinase activity in the anti-FAK immunoprecipitates represented less than 5% of the PI 3-kinase activity observed in the anti-phosphotyrosine immunoprecipitates. Based on these results, it does not appear that FAK plays a major role in the activation of PI 3-kinase in response to hep I.

PI 3-kinase and 3-phosphorylated phosphoinositides have been demonstrated to play an important role in the reorganization of the actin cytoskeleton, particularly membrane ruffling (16–18). However, this is the first demonstration that PI 3-kinase is involved in the disassembly of focal adhesions. Focal adhesion disassembly stimulated by TSP1 or hep I does not involve membrane ruffling, suggesting that PI 3-kinase can have multiple, distinct effects on cytoskeletal organization. Furthermore, insulin, which stimulated PI 3-kinase activity in these experiments, neither caused membrane ruffling nor focal adhesion disassembly (data not shown). Therefore, it appears, at least under these experimental conditions, that PI 3-kinase activity alone is not sufficient for focal adhesion disassembly or membrane ruffling. Localization and extent of activity may account for some of the differences observed in cellular events. Activation of other signaling pathways in parallel may also be required to elicit certain responses. Understanding the mechanisms by which specific signaling pathways result in cellular events is a question fundamental to cell signaling.
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