Thrombospondin Stimulates Focal Adhesion Disassembly through Gι- and Phosphoinositide 3-Kinase-dependent ERK Activation*

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The matricellular protein thrombospondin (TSP) stimulates stress fiber and focal adhesion disassembly through a sequence (hep I) in its heparin-binding domain. TSP/hep I signals focal adhesion disassembly by binding cell surface calreticulin (CRT) and activating phosphoinositide 3-kinase (PI3K). However, other components of this signaling pathway have not been identified. We now show that TSP induces focal adhesion disassembly through activation of pertussis toxin (PTX)-sensitive G proteins and ERK phosphorylation. PTX pretreatment inhibits TSP/hep I-mediated focal adhesion disassembly as well as PI3K activation. In addition, membrane-permeable Gαι- and Gβγ-blocking peptides inhibit hep I-mediated focal adhesion disassembly. Hep I stimulates a transient increase in ERK activation, which is abrogated by both PTX and PI3K inhibitors. Inhibiting ERK activation with MEK inhibitors blocks hep I-mediated focal adhesion disassembly, indicating that ERK activation is required for cytoskeletal reorganization. G protein signals and ERK phosphorylation are induced by TSP binding to cell surface CRT, because CRT null mouse embryonic fibroblasts (MEF) fail to stimulate ERK phosphorylation in response to TSP/hep I treatment. These data show that Gι protein and ERK, in concert with PI3K, are stimulated by TSP-CRT interactions at the cell surface to induce de-adhesive changes in the cytoskeleton.

Cell adhesion is a key regulator of cellular physiology and pathophysiology, affecting the ability of a cell to proliferate, migrate, and even survive (1, 2). Thus, modulation of cell adhesion potentially affects diverse aspects of cell behavior. One adhesion modulatory signal comes from a family of extracellular matrix proteins, termed matricellular proteins, which primarily stimulate anti-adhesive signals. The matricellular proteins, thrombospondin (TSP), tenascin-C, and SPARC, can support varying degrees of cell adhesion (3, 4). However, a feature of these matricellular proteins is that they reduce cellular adhesiveness to a state of intermediate cell adhesion (3). This entails disassembly of focal adhesions, characterized by unbinding of actin stress fibers and selective depletion of certain focal adhesion proteins, including vinculin and α-actinin (3, 5, 6). However, in the intermediate adhesive state, integrins remain clustered and cell spreading is not appreciably altered (3). The functional significance of this adhesive state has yet to be defined, but it is reasonable to suggest that cells in this state have altered tensional forces. Current data suggest that focal adhesion disassembly can modulate the migratory capacity of the cell, affect cellular apoptosis, and induce alterations in gene expression (3). Despite the potential physiologic implications of cellular de-adhesion, very little is currently known about the cell surface receptors and intracellular signaling pathways that propagate focal adhesion disassembly in response to the matricellular proteins.

Thrombospondin (TSP) is a large (180 kDa), homotrimeric, multidomain extracellular matrix glycoprotein. TSP binds several receptors on the cell surface, including heparan sulfate proteoglycans, calreticulin (CRT), CD36, integrin-associated protein (IAP), as well as the αιβι and αιβι integrins, making TSP’s role in physiology and pathophysiology complex (7, 8). The sequence in TSP responsible for inducing focal adhesion disassembly has been mapped to a 19-amino acid sequence in the N-terminal heparin-binding domain of TSP, termed the hep I sequence, because it lies within the first heparin-binding sequence of this domain (9). Early work on the signaling of TSP-mediated focal adhesion disassembly illustrated that basal levels of PKG were required for this process (10). More recent work showed that TSP and hep I induce focal adhesion disassembly by binding to cell surface CRT and activating PI3K (11, 12). The hep I sequence of TSP binds to cell surface CRT, and this binding is necessary for hep I-mediated PI3K activation and focal adhesion disassembly (12). Although best known as an ER resident chaperone protein, CRT localizes to other cellular compartments, such as the cytosol and on the cell surface (13–15). In addition to TSP, cell surface CRT propagates cellular responses to fibrinogen and glycosylated laminin, suggesting this surface form of CRT has a functional role as a cell surface receptor (14, 15). Although CRT is not a transmembrane protein, there is evidence that cell surface CRT engages intracellular signaling pathways. Cho et al. (16, 17) reported that cell surface CRT transmits the effects of an anti-microbial peptide on neutrophils and monococytes. The effects of this anti-microbial peptide are sensitive to pertussis toxin (PTX), a selective inhibitor of the Gi subclass of heterotrimeric G proteins, suggest-
ing that CRT may act in conjunction with a G protein-coupled receptor to propagate intracellular signals. Pertussis toxin-sensitive G proteins are logical targets for the TSP-mediated focal adhesion disassembly response through CRT, because PTX-sensitive G proteins have previously been shown to stimulate focal adhesion and stress fiber disassembly in response to urokinase-type plasminogen activator and fibroblast-derived motility factor (18, 19). Two of the most common pathways stimulated downstream of PTX-sensitive G proteins are the PI3K and ERK signaling pathways (20, 21). Although PI3K is known to play a role in regulating cell adhesion by TSP, little is known about ERK’s role in TSP signaling.

Extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 comprise the mitogen-activated protein kinase (MAPK) family of proteins (22). ERK is activated by diverse stimuli and has been implicated in a wide range of cellular functions, including proliferation, migration, and survival (23). Although the role of adhesion in regulation of the ERK pathway has been widely studied, little is known concerning the effect of ERK signaling on adhesion itself. Signaling through the ERK pathway stimulates a decrease in integrin affinity, suggesting a negative feedback loop on cell adhesion (24). In addition, ERK has been shown to play a role in EGF-mediated focal adhesion disassembly, as well as focal adhesion disassembly induced by oncogenic Ras (25, 26).

In these current studies, we characterize the role of PTX-sensitive G proteins and the ERK pathway in TSP-mediated focal adhesion disassembly. We show that TSP induces G-dependent PI3K and ERK activation, both of which are required for TSP-mediated focal adhesion disassembly. Furthermore, these data report, for the first time, the ability of cell surface CRT to mediate ERK activation.

EXPERIMENTAL PROCEDURES

Materials—The following items were purchased: Dulbecco’s modified Eagle’s medium (DMEM, Cell-Gro, Mediatech, Herndon, VA), fetal bovine serum (FBS, HyClone Laboratories), 500 μg/ml trypsin, 2 mM EDTA (Invitrogen), prestained molecular weight markers (Bio-Rad), and a cholineluminescence Pierce Enzyme Detection kit (Wild-type), K42 (CRT-knockout), and K42 (CRT-rescued)) were a gift of Dr. Marek Michalak, University of Alberta, Edmonton, Alberta, Canada (28). Growth conditions were the same as described for BAE cells. focal Adhesion Assay—Focal adhesion assays were performed as previously described (6). Briefly, BAE and MEF cells grown over confluent glass coverslips in DMEM with 10% FBS. After a 20 min incubation, cells were −70% to 80% confluent. Cells were then washed once with serum-free DMEM and incubated in serum-free DMEM for 30 min. Cells were then treated with either DMEM, hep I (1 μM), or TSP (10 μg/ml = 78 μM monomer), fixed with 3% glutaraldehyde, and examined using a Zeiss Axiovert 10 equipped for interference reflection microscopy as either positive or negative for the presence of focal adhesions, with cells containing at least five focal adhesions considered positive. At least 300 cells were evaluated for each condition.

Cell Lysate Preparation—To prepare whole cell lysates from BAE and MEF cells grown in six-well plates, medium was removed from cells and 100 μl of SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% w/v bromphenol blue) was added to each well. Following lysis is SDS sample buffer, lysates were harvested with cell scrapers and collected in Eppendorf tubes. Lysates were sonicated for 15 s to shear DNA and lower viscosity. Lysates were then boiled, centrifuged, and frozen at −20 °C until gel electrophoresis was performed.

ERK Kinase Assay—BAE cells were grown to near confluence and treated with hep I (1 μM) for various time points. Cells were then harvested by addition of lysis buffer (with 25 mM NaF and 1 mM Na3VO4, 1% Triton X-100, 0.5% Nonidet P-40, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) was added. Cells were scraped, collected in Eppendorf tubes, and pre-cleared by centrifugation. Supernatants were incubated with PY20 (10 μg/ml) antibodies for 2 h on ice. Protein A-Sepharose was added for 1 h at 4 °C with shaking. Immunoprecipitates were washed three times with lysis buffer and twice in kinase buffer (10 μM HEPES, pH 7.2, 20 mM β-glycerophosphate, 0.8 mM Na3VO4, and 30 mM NaCl). Lipids were prepared by adding 400 μl of kinase buffer with 3.5 mM diithiothreitol to a pre-dried equal mixture of NaHCO3 and phosphatidylserine, to achieve a final 1.5 μM concentration of each lipid. Kinase buffer was removed from immunoprecipitates, and 20 μl of lipids was added to each tube and incubated for exactly 10 min at 37 °C. Next, 20 μl of reaction buffer (kinase buffer containing 17.5 μM ATP, 25 μCi of [32P]ATP/sample, and 17.5 μM MgCl2) was added to each tube and incubated at 37 °C for 10 min. The reaction was stopped by adding 160 μl of a 1:1 methanol/ chloroform solution. Lipids were extracted by adding 80 μl of HCl to each tube and centrifuging to separate the phases. The lower phase was removed and lipids were separated by TLC on Silica Gel 60 plates pre-coated with 1% potassium oxalate. The phases were separated by liquid scintillation assay. Samples were analyzed for [32P]ATP using a Zeiss Axiovert 10 equipped for interference reflection microscopy. Cells were scored as either positive or negative for the presence of focal adhesions, with cells containing at least five focal adhesions considered positive. At least 300 cells were evaluated for each condition.

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**RESULTS**

**TSP and Hep I Stimulate Focal Adhesion Disassembly through Pertussis Toxin-sensitive Heterotrimeric G Proteins**—We previously characterized a role for cell surface CRT in TSP/hep I-mediated focal adhesion disassembly and PI3K activation, suggesting that CRT is acting as a receptor for the hep I sequence of TSP (12). However, CRT is not a transmembrane protein and most likely associates with a transmembrane protein to generate an intracellular signal. Because cell surface CRT has been shown to signal through a G protein-dependent mechanism in neutrophils and monocytes (16, 17), we sought to determine what role heterotrimeric G proteins might play in TSP-induced focal adhesion disassembly.

Pertussis toxin catalyzes the ADP-ribosylation and inactivation of the G,i, subclass of heterotrimeric G proteins (29). To determine if pertussis-toxin sensitive G proteins are involved in TSP/hep I-mediated focal adhesion disassembly, we observed the effect of pertussis toxin (25 ng/ml for 12 h) pretreatment on TSP and hep I-mediated focal adhesion disassembly. Pretreatment of BAE cells with pertussis toxin blocked the ability of both TSP and hep I to stimulate a decrease in the percentage of focal adhesion-positive cells as measured by IRM (Fig. 1A). Pertussis toxin did not affect the basal level of focal adhesion-positive cells. The active sequence (TNfIIIAD) of tenascin-C (27), another matricellular protein, retained the ability to induce focal adhesion disassembly in the pertussis toxin-treated cells (Fig. 1B), suggesting the effect of pertussis toxin is specific for TSP. In addition, pertussis toxin inactivated by boiling did not inhibit TSP/hep I-mediated focal adhesion disassembly (data not shown). Cholera toxin, which activates the G,i, subclass of heterotrimeric G proteins, had no effect on focal adhesion disassembly (data not shown). Cholera toxin, which activates the G,i, subclass of heterotrimeric G proteins (29), was specific for TSP. In addition, pertussis toxin inactivated by boiling did not inhibit TSP/hep I-mediated focal adhesion disassembly (data not shown). Cholera toxin, which activates the G,i, subclass of heterotrimeric G proteins, had no effect on focal adhesion disassembly (data not shown).

Since activation of PI3K by TSP/hep I was shown to be essential for focal adhesion disassembly (11), we also sought to determine whether pertussis toxin could inhibit the ability of TSP/hep I to activate PI3K. BAE cells were serum-deprived in the presence or absence of 25 ng/ml PTX for 12 h, stimulated with hep I, TSP, or a modified hep I peptide, and the resulting PI3K activity was assessed. Although pertussis toxin slightly inactivated PI3K activity, pertussis toxin treatment significantly inhibited the ability of both TSP and hep I to stimulate PI3K activation (Fig. 2). A modified form of the hep I peptide, with the essential lysines at positions 24 and 32 converted to alanine residues, did not stimulate PI3K activation under either condition. Pertussis toxin does not generally affect PI3K activity, because insulin stimulated PI3K activation was not inhibited by pertussis toxin (data not shown). These data provide evidence for the involvement of heterotrimeric G proteins in TSP/hep I-mediated focal adhesion disassembly.

BAE cells contain only two known pertussis toxin-sensitive G proteins, Ga32 and Ga33 (30). To further characterize the involvement of these proteins in TSP and hep I-mediated focal adhesion disassembly, we employed a membrane-permeable peptide approach to specifically block signaling through Ga32, Ga33, and their associated Gβγ subunits. The C-terminal 10 amino acids from the Gα subunits have previously been demonstrated to specifically block the G protein-receptor interaction (31). Thus, the C-terminal 10 amino acids from Ga32 and Ga33 were produced coupled to the membrane-permeable sequence from Kaposi fibroblast growth factor. This approach has proven successful in blocking G protein signaling, delivering the inhibitory peptides into every cell type tested to date (32, 33). In addition, a Gβγ-sequestering 28-amino acid peptide from the C terminus of phosducin-like protein, a known Gβγ signaling inhibitor, was also produced and coupled to the membrane-permeable sequence (32, 33). BAE cells were incubated for 1 h with the various peptides (1 μM), treated with DMEM or hep I for 30 min, and the percentage of focal adhesion-positive cells was assessed by interference reflection microscopy. Pretreatment with both the Ga32 inhibitory peptide and the Gβγ inhibitory peptides was able to inhibit hep I-mediated focal adhesion disassembly (Fig. 3). Interestingly, the Ga32 inhibitory peptide was not able to block this effect, although its functional sequence differs from that of the Ga32 inhibitory peptide in only 2 of the 10 amino acids. The membrane-permeable sequence alone had no effect on hep I-induced focal adhesion disassembly, suggesting that the effect is specific for the G protein-inhibitory sequence.
TSP Stimulates an Increase in ERK Phosphorylation—Pertussis toxin-sensitive G proteins have been shown to stimulate activation of the ERK signaling pathway in response to a variety of agonists (20). Because ERK signaling stimulates focal adhesion disassembly in other systems (25, 26), the role of the ERK pathway in TSP-mediated focal adhesion disassembly was examined. It was first determined whether TSP/hep I is able to stimulate signaling through ERK. BAE cells were treated with hep I for various time points, and ERK activation was assessed by Western blotting with an antibody specific for phosphorylated ERK. Hep I treatment stimulated a transient increase in ERK phosphorylation in BAE cells (Fig. 4A). Surprisingly, treatment with hep I for 2 min caused the levels of phosphorylated ERK to drop to ~80% of baseline values, suggesting that some of the immediate responses to hep I might be due to decreased signaling through the ERK pathway. However, by 5 min, ERK phosphorylation was increased and activation peaked at ~2.6-fold above baseline values after 10 min of hep I stimulation. This decrease and subsequent increase in phosphorylated ERK was not seen in cells treated with DMEM alone, suggesting the response is specific for hep I and is not an artifact of the procedure. Following the initial peak in ERK activation, levels of phosphorylated ERK decreased slightly at 30 min, followed by a gradual increase in ERK phosphorylation, which was sustained for at least 2 h following stimulation. The time course for ERK phosphorylation correlates with TSP/hep I-mediated focal adhesion disassembly, with disassembly becoming apparent at 5 and 10 min after treatment and maximal by 30 min (9). In addition, the effect of hep I on ERK activity was also assessed using an immunoprecipitation kinase assay. BAE cells were treated with hep I (1 μM) for various time points, and total ERK protein was immunoprecipitated. Resulting immunoprecipitates then underwent an in vitro kinase assay by successive incubations with phosphatidylinositol 4,5-bisphosphate (PIP₃) and [³²P]ATP. Phosphorylated lipids were separated by thin layer chromatography, detected by autoradiography, and analyzed using densitometry. Results are the mean arbitrary absorbance units for each treatment ± S.D. (n = 3–5). *, p < 0.05 as compared with DMEM.

FIG. 2. TSP/hep I-mediated PI3K activation is PTX-sensitive. BAE cells were grown to near confluence in 100-mm² tissue culture plates. Cells were then serum-deprived overnight in either 0.2% FBS or 0.2% FBS + 25 ng/ml pertussis toxin. Cells were then treated with either DMEM, hep I (1 μM), TSP (78 nM), or modified hep I (1 μM) for 30 min. Cells were lysed and immunoprecipitated with anti-phosphotyrosine (PY20) antibodies. Immunoprecipitates then underwent an in vitro lipid kinase assay by successive incubations with phosphatidylinositol 4,5-bisphosphate (PIP₃) and [³²P]ATP. Phosphorylated lipids were separated by thin layer chromatography, detected by autoradiography, and analyzed using densitometry. Results are the mean arbitrary absorbance units for each treatment ± S.D. (n = 3–5). *, p < 0.05 as compared with DMEM.

FIG. 3. Hep I-mediated focal adhesion disassembly is sensitive to Gα₂ and Gβγ inhibition. BAE cells were grown to near confluence on glass coverslips. Cells were washed and treated with the G protein inhibitory peptides (1 μM) MPS, MPS-Gα₁₂, MPS-Gα₁₃, or MPS-Phos for 1 h, followed by treatment with either DMEM or hep I (1 μM) for 30 min. Cells were fixed in glutaraldehyde, washed, and mounted on glass slides, and the percentage of focal adhesion-positive cells was determined by IRM. Results are the mean number of cells positive for focal adhesions ± S.D. (n = 3–5). *, p < 0.01; **, p < 0.001; and ***, p < 0.0001 as compared with DMEM.

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To ensure that hep I is signaling similarly to intact TSP, the ability of TSP to stimulate ERK phosphorylation was also examined. BAE cells were treated with hep I (100 nM), modified hep I (100 nM), TSP (78 nM) for 10 min, or insulin (100 nM) for 5 min. TSP stimulated ERK phosphorylation to a similar extent as hep I, whereas the modified hep I peptide did not stimulate significant ERK activation (Fig. 4C). This level of TSP/hep I-induced ERK phosphorylation was similar to that seen with insulin stimulation, suggesting that the increase in ERK phosphorylation is significant.

TSP-mediated Focal Adhesion Disassembly Is Sensitive to MEK Inhibitors—Given that the hep I sequence of TSP stimulates ERK activation, we then determined whether signaling through the ERK pathway is involved in focal adhesion disassembly. BAE cells were incubated with the MEK inhibitors PD98059 (50 μM) and U0126 (10 μM) for 1 h then treated with hep I for 30 min, and the percentage of focal adhesion-positive cells was assessed using interference reflection microscopy. Both the PD98059 and U0126 compounds inhibited hep I-mediated focal adhesion disassembly, suggesting that ERK phosphorylation is necessary for this process (Fig. 5A). Neither inhibitor showed a significant effect on the basal number of focal adhesion-positive cells. In addition, the p38 inhibitor SB202190 also blocked TSP-mediated focal adhesion disassembly, although p38 activation was not detected in response to TSP (data not shown), suggesting that the other MAPK pathways may also play a role in propagating this response. The JNK inhibitor SP600125 caused focal adhesion instability, and thus could not be used to test whether JNK signaling was required for TSP-mediated focal adhesion disassembly (data not shown). To assess ERK’s role in focal...
adhesion disassembly by other matricellular proteins, the effect of blocking MEK signaling on tenasin-induced focal adhesion disassembly was determined. Consistent with the observed effects on TSP-induced focal adhesion disassembly, MEK inhibition was also able to block tenasin-induced focal adhesion disassembly, suggesting that the ERK pathway may play a pivotal role in multiple focal adhesion disassembly signaling pathways (Fig. 5B).

![Fig. 4. TSP/hep I stimulates transient ERK phosphorylation.](image)

A. BAE cells were grown to near confluence overnight in six-well plates in 1% FBS. Cells were then serum-starved in serum-free DMEM for 2 h. Cells were treated with either DMEM or hep I (1 μM) for 0, 2, 5, 10, 15, 30, 60, and 120 min. Cells were lysed with SDS sample buffer, separated by SDS-PAGE, transferred to PVDF membranes, and incubated with rabbit anti-phospho-ERK antibodies. HRP-conjugated goat anti-rabbit antibodies were added, and proteins detected through chemiluminescence. Levels of phospho-ERK were determined through densitometry, and normalized to total ERK levels. Results are the mean arbitrary absorbance units for each treatment ± S.D. ERK phosphorylation at 10 min was significant at *p < 0.001, 15 min at *p < 0.0001, and 60 min at *p < 0.01 (n = 4–7). B. BAE cells were grown to near confluence overnight in six-well plates in 1% FBS. Cells were then serum-starved in serum-free DMEM for 2 h. Cells were treated with either DMEM or hep I (1 μM) for 0, 5, 10, 15, 30, 60, and 120 min. Cells were lysed and immunoprecipitated with anti-MAPK 1/2 antibodies. Immunoprecipitates then underwent an in vitro kinase assay by successive incubations with dephosphorylated myelin basic protein (MBP) and ATP. Samples were then separated by SDS-PAGE, immunoblotted with phospho-MBP-specific antibodies, and detected by autoradiography. Results shown are representative of three separate experiments. C. BAE cells were grown to near confluence overnight in six-well plates in 1% FBS. Cells were then serum-starved in serum-free DMEM for 4 h. Cells were treated with either DMEM, hep I (100 nM), TSP (78 nM), or modified hep I (100 nM) for 10 min or insulin (100 nM) for 5 min. Cells were lysed with SDS sample buffer, separated by SDS-PAGE, transferred to PVDF membranes, and incubated with rabbit anti-phospho-ERK antibodies. HRP-conjugated goat anti-rabbit antibodies were added, and proteins were detected through chemiluminescence. Levels of phospho-ERK were determined through densitometry and normalized to total ERK levels. Results are the mean arbitrary absorbance units for each treatment ± S.D. (n = 3–4). *, *p < 0.01, **p < 0.001 as compared with DMEM.

![Fig. 5. ERK activation is required for TSP/hep I-mediated focal adhesion disassembly.](image)

A. BAE cells were grown to near confluence on glass coverslips. Cells were washed and treated with the MEK inhibitors PD98059 (50 μM) and U0126 (10 μM) or the p38 inhibitor SB202190 (1 μM) for 1 h, followed by treatment with either DMEM or hep I (1 μM) for 30 min. Cells were fixed in glutaraldehyde, washed, and mounted on glass slides, and the percentage of focal adhesion-positive cells was determined by IRM. Results are the mean number of cells positive for focal adhesions ± S.D. (n = 4–6). *, *p < 0.01 as compared with baseline. B. BAE cells were grown to near confluence on glass coverslips. Cells were washed and treated with either DMEM or U0126 (10 μM) for 1 h, followed by treatment with either DMEM or TNfnIII-A-D (30 μg/ml) for 30 min. Coverslips were prepared for IRM and focal adhesions assessed as described in A. Results are the mean number of cells positive for focal adhesions ± S.D. (n = 4–5). *, *p < 0.01 as compared with DMEM.

**ERK Activation in Response to Hep I Requires PTX-sensitive G Proteins, PI3K, and Cell Surface CRT**—To better understand the role of ERK activation in TSP-mediated focal adhesion disassembly, we determined where ERK activation occurs with respect to other known proteins in the TSP/hep I-induced signaling pathway. Current data show that TSP/hep I signals through cell surface CRT and pertussis toxin-sensitive heterotrimeric G proteins, resulting in PI3K activation and subsequent focal adhesion disassembly. The effect of blocking these individual signals on hep I-mediated ERK phosphorylation was determined. BAE cells serum-starved for 4 h in the presence or absence of pertussis toxin (100 ng/ml for 4 h), the PI3K inhibitors wortmannin (2.5 nM for 30 min) and LY294002 (5 μM for 30 min), or the MEK inhibitor U0126 (10 μM for 1 h), were treated with hep I (1 μM) for 10 min and analyzed for ERK activation as previously described. Pertussis toxin completely inhibited the ability of hep I to stimulate ERK phosphorylation (Fig. 6). Inhibition of PI3K, either with wortmannin or LY294002, resulted in a significant, but incomplete, inhibition of hep I-mediated ERK phosphorylation, suggesting that ERK
were treated with either 2.5 nM wortmannin or 5 μM LY294002. In the last hour of serum starvation, some cells received 10 μM U0126. In the last half hour of serum starvation, some BAE cells were treated with either 2.5 μM wortmannin or 5 μM LY294002. Following serum starvation, cells were treated for 10 min with either DMEM or hep I (1 μM). Cells were lysed with SDS sample buffer, separated by SDS-PAGE, transferred to PVDF membranes, and incubated with rabbit anti-phospho-ERK antibodies. HRP-conjugated goat anti-rabbit antibodies were added, and proteins were detected through chemiluminescence. Levels of phospho-ERK were determined through densitometry and normalized to total ERK levels. Results are the mean arbitrary absorbance units for each treatment ± S.D. (*p < 0.01 as compared with DMEM.

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Total ERK levels were significantly lowered background levels of phosphorylated ERK. Although the wild-type MEFs showed a significant activation of ERK in response to hep I, the CRT null MEFs failed to activate ERK in response to hep I treatment. In addition, transfection of null cells with CRT rescued the ability of hep I to activate ERK (Fig. 7A). Interestingly, the CRT null MEFs had a significantly reduced basal level of phosphorylated ERK, whereas there was an increase in total ERK protein in the CRT null MEFs as compared with both wild-type and rescued cells, indicating that only a small percentage of ERK in CRT null MEFs is phosphorylated (Fig. 7B). This might suggest that the ER chaperone role of CRT is necessary to process some intermediary in the ERK signaling pathway. However, tenasin-C, another member of the matricellular family of extracellular matrix proteins that does not utilize CRT-dependent signaling, also induces ERK phosphorylation (Fig. 7C) and stimulates focal adhesion disassembly through a MEK-dependent process. Because tenasin-C is able to stimulate both ERK activation and focal adhesion disassembly in the CRT null MEFs, the reduced levels of basal ERK phosphorylation in the CRT null cells are not sufficient to inhibit ERK activation or inhibit focal adhesion disassembly. Together, these data show that cell surface CRT is required for hep I-mediated ERK activation.

DISCUSSION

TSP had been shown to stimulate focal adhesion disassembly through the interaction of its hep I sequence with cell surface CRT and activation of PI3K (9, 11, 12). We now show that the
activation of pertussis toxin-sensitive G proteins, such as \( \text{G}_{\text{i2}} \) and \( \text{G}_{\beta\gamma} \). These G proteins then activate PI3K and stimulate ERK phosphorylation primarily through a PI3K-dependent mechanism. Lastly, activation of both PI3K and ERK are necessary to stimulate TSP/hep I-mediated focal adhesion disassembly.

The hep I sequence of TSP stimulates the activation of pertussis toxin-sensitive G proteins, which then signal to PI3K and ERK, both of which are necessary to drive focal adhesion disassembly (Fig. 8). Although PI3K activity was previously shown to be necessary for TSP-mediated focal adhesion disassembly, this is the first work identifying G proteins and ERK signaling as required components of this pathway. This work presents new insights into the mechanisms employed by TSP to induce the characterized changes in both focal adhesion and cytoskeletal architecture and presents further evidence for the roles of the \( \text{G}_i \) subclass of heterotrimeric G proteins and the ERK signaling pathway in regulating cellular de-adhesion.

A role for pertussis toxin-sensitive G proteins and ERK in TSP signaling has been reported (34–37). The C-terminal domain of TSP interacts with IAP and activates pertussis toxin-sensitive G proteins, leading to increases in integrin affinity and cell adhesion (34). Although signaling through IAP activates pertussis toxin-sensitive G proteins, the IAP-binding peptides from TSP do not stimulate focal adhesion disassembly unless given at very high (100 \( \mu \text{M} \)) concentrations. Wang et al. (36) demonstrated that TSP stimulates IAP-dependent ERK inactivation in SMCs. In contrast, Gahtan et al. (37) showed that TSP stimulates ERK activation in smooth muscle cells (SMC), although the domain of TSP-signaling ERK activation was not shown. These data suggest that although the C and N termini of TSP can both signal through PTX-sensitive pathways, the downstream targets and physiologic consequences of these signals differ.

The ability of CRT, a peripheral membrane protein, to stimulate these intracellular signals is unusual, but not unique (16, 17). Interestingly, the matricellular protein tenascin-C also stimulates focal adhesion disassembly through binding to a peripheral calcium-binding protein, annexin II, on the cell surface (38). GPCRs are known to bind to a wide variety of agonists, making them good potential targets for signaling through protein kinase C, associated with the formation of actin stress fibers and an increase in focal adhesion formation (42). In addition, the \( \text{G}_{12/13} \) family of G proteins stimulates RhoGEF activity, inducing Rho activation, which stimulates stress fiber and focal adhesion formation (43). Although signaling through the \( \text{G}_q \) and \( \text{G}_{12/13} \) subtypes of G proteins has long been associated with an increase in actin stress fibers and focal adhesions, signaling through the \( \text{G}_i \) subclass is consistent with focal adhesion disassembly responses. Pertussis toxin-sensitive G proteins stimulate focal adhesion disassembly in response to urokinase-type plasminogen activator as well as to fibroblast-derived motility factor (18, 19). In addition, the matricellular protein SPARC also stimulates focal adhesion disassembly through pertussis toxin-sensitive G proteins. Tenascin-C, however, signals focal adhesion disassembly through a PTX-insensitive mechanism, suggesting both PTX-dependent and -independent pathways can mediate focal adhesion disassembly. The exact role of individual G protein subunits on cell adhesion has been difficult to determine, because many receptors couple to multiple G protein subunits.

The use of membrane-permeable peptides to inhibit specific signaling events is becoming increasingly popular, especially in studies of G protein signaling (32, 33). The G protein inhibitory peptide mimics the major GPCR-binding region on \( \text{G}_\alpha \) subunits, thus preventing specific receptor-G protein binding and activation (31). The finding that the \( \text{G}_{\alpha_q} \) sequence, blocks hep I-mediated focal adhesion disassembly was unexpected, because many GPCRs couple to both G proteins, although often with differing affinities (44, 45). The lack of effect with the \( \text{G}_{\alpha_q} \) peptide does not appear to be due to a decreased affinity, however, because this peptide when tested at a 20 \( \mu \text{M} \) higher concentration also failed to block focal adhesion disassembly. Preferential coupling of GPCRs to either \( \text{G}_{\alpha_2} \) or \( \text{G}_{\alpha_1} \) is not unprecedented, as the interleukin-8 receptor only to \( \text{G}_{\alpha_2} \) and not to \( \text{G}_{\alpha_1} \) (46).

Interplay between pertussis toxin-sensitive G proteins, ERK, and PI3K is well established (47–49). Some PI3K catalytic subunits, such as the p110\( \beta \) and p110\( \gamma \) isoforms, can be activated by direct interaction with G protein \( \beta \gamma \) subunits (50). However, we have evidence that TSP/hep I activates the p85\( \gamma \) isoforms, equally, implying that direct interactions between G protein subunits and PI3K are not the main activation mechanism (data not shown). Signaling through \( \text{G}_i \) as well as \( \text{G}_q \) induces transactivation of a number of tyrosine-phosphorylated scaffolding proteins, including growth factor receptors and components of focal adhesions, which stimulate activation of PI3K and the ERK pathway (47). Thus, G protein-dependent stimulation of tyrosine phosphorylation is the most likely mechanism for TSP-induced PI3K and ERK activation. PI3K is important in ERK activation, ras stimulation, and phosphorylation of MEK through PI3K-stimulated PAK activity (48, 49, 51). Greenwood et al. (52) demonstrated that the lipid products of PI3K alone can be sufficient for focal adhesion disassembly. PIP\(_2\), the major product of PI3K, binds \( \alpha \)-actinin and causes it to dissociate from stress fibers and focal adhesions, inducing disassembly of these structures (52). However, PI3K activity alone is not sufficient to stimulate focal adhesion disassembly in response to TSP, because activation of ERK and of FAK, which occurs independ-

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3 M. A. Pallero and J. E. Murphy-Ullrich, unpublished data.

4 C. E. Pedraza, A. W. Orr, M. A. Pallero, D. K. Strickland, and J. E. Murphy-Ullrich, manuscript in preparation.
ently of PI3K,5 is required for TSP-mediated focal adhesion disassembly. This discrepancy can likely be attributed to differing levels of PI3P and localization of PI3K activity under varying conditions. However, our data are consistent with Wennstrom et al. (49) who showed that PI3K plays a permissive role in ERK activation, but that it is not sufficient to induce ERK activation alone.

One of the key activators of the ERK pathway in adherent cells is the formation of cell-extracellular matrix adhesions, which stimulate ERK phosphorylation through a number of mechanisms (53). In addition, the presence of these adhesive structures is a prerequisite for ERK activation by a variety of stimuli (54). Thus, cell adhesion plays an important role in regulating signaling through the ERK pathway. Recent data, including the work presented herein, suggest that the ERK pathway might also act as a negative regulator of cell adhesion. This regulation appears to occur both at the level of integrin activation and organization of focal adhesions. Activation of the ras/raf pathway stimulates a decrease in integrin binding affinity (25). Focal adhesion disassembly in response to TSP, tenascin-C, and EGF all require ERK activation, suggesting that ERK might be a common effector for signaling focal adhesion disassembly. However, platelet-derived growth factor-inducing focal adhesion disassembly has previously been shown to be ERK-independent, suggesting that ERK is not required for all focal adhesion labilizing stimuli (55). In addition, Glading et al. (56) showed that EGF induces focal adhesion disassembly through ERK-dependent calpain activation. Consistent with this result, the calpain inhibitor MDL was also able to block TSP/pI3P-mediated focal adhesion disassembly, suggesting that EGF and TSP may stimulate focal adhesion disassembly through similar pathways (data not shown).

The physiologic consequences of TSP-mediated focal adhesion disassembly have yet to be determined, although evidence suggests that this response might be important in modulation of cell migration. Focal adhesion disassembly correlates with an increase in cell migration in several models (1, 57). In addition, pertussis toxin-sensitive G proteins, PI3K, and ERK have all shown to play a role in stimulating cell migration (58–60). Future work concerning the effect of HPI signaling on endothelial cell migration will provide further insights into TSP biology.

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