SHP2 Association with VE-Cadherin Complexes in Human Endothelial Cells Is Regulated by Thrombin*

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Thrombin-mediated changes in endothelial cell adherens junctions modulate vascular permeability. We demonstrate that the nonreceptor protein-tyrosine phosphatase SHP2 co-precipitates with VE-cadherin complexes in confluent, quiescent human umbilical vein endothelial cells. Ligand-binding blots using a SHP2-glutathione S-transferase fusion peptide established that SHP2 associates selectively with β-catenin in VE-cadherin complexes. Thrombin treatment of human umbilical vein endothelial cells promotes SHP2 tyrosine phosphorylation and dissociation from VE-cadherin complexes. The loss of SHP2 from the cadherin complexes correlates with a dramatic increase in the tyrosine phosphorylation of β-catenin, γ-catenin, and p120-catenin complexed with VE-cadherin. We propose that thrombin regulates the tyrosine phosphorylation of VE-cadherin-associated β-catenin, γ-catenin, and p120-catenin by modulating the quantity of SHP2 associated with VE-cadherin complexes. Such changes in adherens junction complex composition likely underlie thrombin-elicited alterations in endothelial monolayer permeability.

Confluent, quiescent endothelial and epithelial cell monolayers form semi-permeable barriers that regulate the transit of agents across the monolayer. This barrier function depends, in part, on the cadherin complexes that form the intercellular junctions. The cadherins are transmembrane proteins that draw neighboring cells together through calcium-dependent, homophilic associations. The stability of these cell-cell junctions is modulated by the cadherins, cytoplasmic proteins that bind directly (β-catenin, γ-catenin, and p120-catenin) or associate indirectly (α-catenin) with the intracellular tail of the cadherin (1–3). The catenins regulate tethering of the cadherin complexes to the actin cytoskeleton and lateral cadherin multimer formation (4, 5). VE-cadherin, an endothelial cell-specific cadherin, localizes at the intercellular adhesion junctions formed by endothelial cells and appears to be responsible for the exclusion of N-cadherin, the other highly expressed cadherin in endothelial cells, from these junctions (6, 7). In proliferating endothelial cells, as in epithelial cells, significant tyrosine phosphorylation of β-catenin, γ-catenin, and p120-catenin can be detected (8, 9). As the cells reach confluence and undergo contact inhibition of proliferation and stabilization of cell-cell junctions, tyrosine phosphorylation of the catenins decreases dramatically. This correlates with the density-dependent increase in protein-tyrosine phosphatases (PTP), specifically at cell-cell junctions where they associate with cadherin complex proteins or platelet endothelial cell adhesion molecule (PECAM-1) (10–12).

Thrombin, a potent activator of endothelial cells, causes increased permeability and intercellular gap formation in confluent endothelial cell monolayers (13–15). Such endothelial barrier dysfunction can be elicited by protein-tyrosine phosphatase inhibitors and can be attenuated by protein-tyrosine kinase inhibitors (16–20). The thrombin-induced increases in monolayer permeability correlate with changes in the composition of the VE-cadherin complexes (14).

We hypothesized that thrombin receptor (protease-activated receptor 1) activation of endothelial cells altered VE-cadherin complexes by regulating the tyrosine phosphorylation of the cadherin-associated catenins. In the following studies we found that the cytosolic protein-tyrosine phosphatase SHP2 (PTP2C, PTP1D, and Syp) was associated with VE-cadherin complexes in confluent, quiescent human umbilical vein endothelial cells (HUVEC) and that SHP2 bound selectively to β-catenin in the VE-cadherin complexes. When HUVEC monolayers were exposed to thrombin, SHP2 became tyrosine phosphorylated by a Src family kinase and dissociated from the VE-cadherin complexes. This regulated dissociation of protein-tyrosine phosphatase SHP2 provides a mechanism for thrombin-induced increased tyrosine phosphorylation of VE-cadherin-associated β-catenin, γ-catenin, and p120-catenin.

EXPERIMENTAL PROCEDURES

Cell Culture—HUVEC were isolated and characterized as described by Jaffe et al. (21). Cells were grown in Medium 199 containing 10 mM Hepes, pH 7.4, 10% fetal calf serum, 1 mM glutamine, 12 units/ml heparin, 100 μg/ml crude endothelial cell growth supplement, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C on fibronectin-coated tissue culture dishes. Cells were used at passages 1–3. For metabolic labeling studies, subconfluent cultures of HUVEC were incubated overnight with 20 μCi/ml [35S]Express™ Methionine/Cysteine Protein Labeling Mix (NEN Life Science Products) in a growth medium with methionine-free, cysteine-free Dulbecco’s modified Eagle’s medium substituted for Medium 199.

Antibodies—Antibodies used were: goat and rabbit polyclonal antibodies to VE-cadherin and SHP2, respectively (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); mouse monoclonal antibody to VE-cadherin clone TEA1/31 (Biosign International, Kennebunk, ME); rabbit polyclonal antibody to α-catenin and mouse monoclonal antibodies to β-catenin clone 6F9 and γ-catenin clone 15F11 (Sigma); mouse monoclonal antibody to p120-catenin clone 98 (Transduction Laboratories, Lexington, KY); mouse monoclonal antibody to phosphotyrosine clone 4G10

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the tyrosine phosphorylation of VE-cadherin-associated units/ml thrombin for 5 min at 37 °C, a significant increase in date (Fig. 1A). Very little tyrosine phosphorylation, even when prepared in the complexes from confluent, quiescent endothelial cells exhibit conformational stability in many cell systems (25–27). The VE-cadherin nin, mM H2O2 in Medium 199 containing 25 mM Hepes at 37 °C for 5 min prior to extraction (8). Detergent Extraction—Monolayers were washed with ice-cold phosphate-buffered saline containing 0.7 mM CaCl2 and 0.5 mM MgCl2 and scraped in Triton X-100 extraction buffer (1% Triton X-100, 10 mM Tris-HCl, pH 7.6, 50 mM NaCl, 30 mM Na3P04, 50 mM NaF, 1 mM Na2VO4, 2 mM CaCl2, 0.2 mM H2O2, 2 mM peptide, 1 mM 50 trans-epoxysuccinyl-leucylamido(4-guanidino)-butane, 1 mM α-prolin, 1 mM g/ml leupeptin, and 0.1 g/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride). After solubilization on ice for 15 min with intermittent vortexing, the extract was microcentrifuged for 10 min, and the supernatant was recovered. Immunoblotting—Samples containing equal amounts of protein were solubilized in Laemmli sample buffer (22), separated by SDS-polyacrylamide gel electrophoresis (8% polyacrylamide) and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). Immunoblots probed with monoclonal antibody 4G10 were blocked and probed in the presence of 5% bovine serum albumin (ICN Biomedicals, Inc., Aurora, OH). All other immunoblots were blocked with 5% milk in Tris-buffered saline, pH 7.5 (TBS), and probed sequentially with primary and secondary antibodies diluted in 0.5% milk in TBS. Detection was by enhanced chemiluminescence (NEL Life Science Products). Immunoprecipitation—Samples, boiled in 1% SDS where indicated, were brought up to 1 ml with Triton X-100 extraction buffer, rotated 1 h at 4 °C with antibody, and then incubated for 2 h or overnight with protein G-agarose (Sigma). The immunoprecipitated proteins were collected by centrifugation, and the pellets washed three times with phosphate-buffered saline containing 0.7 mM CaCl2, 0.5 mM MgCl2, 1 mM Na2VO4, and 0.2 mM H2O2. Pellets were resuspended in sample buffer and analyzed as described above for immunoblotting. SHP2 Fusion Peptide-Agarose—Ligand binding precipitations were performed in the same manner as immunoprecipitations with SHP2-GST fusion peptide conjugated to agarose (Santa Cruz Biotechnology, Inc.) substituted for antibody and protein G-agarose. Ligand Binding Blot—Extracts, boiled in 1% SDS, were subjected to the immunoprecipitation protocol, separated by SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membrane. The polyvinylidene difluoride membrane was blocked in 5% bovine serum albumin in TBS for 4 h and then incubated in 5% bovine serum albumin-TBS containing 1 μg/ml SHP2-GST fusion peptide (Santa Cruz Biotechnology, Inc.) overnight at 4 °C. The membrane was washed extensively in TBS, incubated with anti-GST antibody (Santa Cruz Biotechnology, Inc.) for 1 h, washed extensively in TBS, and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. The membranes were washed extensively in TBS, and bound GST fusion peptide was detected by enhanced chemiluminescence.

RESULTS AND DISCUSSION
Thrombin causes increased intercellular gap formation and monolayer permeability in endothelial cells (13–16). We examined whether thrombin, as other cytokines (e.g. VEGF), altered endothelial cell adherens junction organization by modifying the tyrosine phosphorylation of β-catenin, γ-catenin, and p120-catenin (23, 24). Such increases in the tyrosine phosphorylation of catenins have been shown to decrease intercellular junctional stability in many cell systems (25–27). The VE-cadherin complexes from confluent, quiescent endothelial cells exhibit very little tyrosine phosphorylation, even when prepared in the presence of the potent tyrosine kinase type II kinase inhibitor pertussis toxin (Fig. 1A, 5 min). When HUVEC were incubated with 2 units/ml thrombin for 5 min at 37 °C, a significant increase in the tyrosine phosphorylation of VE-cadherin-associated β-catenin, γ-catenin, and p120-catenin was observed (Fig. 1A, 5 min; Fig. 1B documents the relative mobilities of the catenins). The thrombin-stimulated phosphorylation was transient, with little phosphotyrosine detectable on cadherin components at 30 min (Fig. 1A, 30 min).

Changes in the tyrosine phosphorylation of the cadherin-associated catenins likely are regulated by the association and/or activity of protein-tyrosine kinases and phosphatases. HUVEC express significant quantities of the nonreceptor PTPs, SHP2, and PTP1B. When we analyzed HUVEC monolayers for the subcellular distribution of SHP2 and PTP1B by indirect immunofluorescence microscopy, only SHP2 localized at the junctions of perversanadate-treated monolayers (data not shown). Others have demonstrated that SHP2 binds by means of its tandem Src homology 2 (SH2) domains to the endothelial cell-cell junctional protein, PECAM-1 when it is tyrosine phosphorylated (28, 29). VE-cadherin and its associated catenins contain consensus binding sites for SH2 domains, so we examined whether SHP2 associated with VE-cadherin complexes. When extracts of confluent, quiescent HUVEC monolayers were immunoprecipitated with VE-cadherin antibody and immunoblotted for SHP2, SHP2 co-precipitated with the VE-cadherin complexes (Fig. 2). If the HUVEC monolayers were incubated with thrombin (2 units/ml) for 5 min prior to extraction, SHP2 association with VE-cadherin complexes was markedly diminished. When HUVEC monolayers were exposed to thrombin for 30 min, SHP2 association with VE-cadherin complexes was not detected. SHP2 disassociation from VE-cadherin complexes in HUVEC was contingent upon thrombin receptor activation, which occurs within 5 min of exposure of HUVEC monolayers to thrombin (15, 30), and SHP2 re-association was detected at later times (after 30 min of continuous exposure to thrombin), consistent with thrombin receptor desensitization. To determine which proteins of the VE-cadherin complex bound SHP2, we performed ligand-biding blots using a GST fusion peptide containing the tandem SH2 domains of SHP2 (SHP2 amino acids 6–213). HUVEC extracts were boiled in 1% SDS to disassemble the cadherin complexes prior to immunoprecipitation with antibodies specific for VE-cadherin, α-catenin, β-catenin, γ-catenin, or p120-catenin. The SHP2-GST fusion peptide consistently associated with β-catenin (Fig. 3). Analogous experiments performed with extracts from metabolically labeled cells indicated that equivalent quantities of all proteins were immunoprecipitated under these conditions except for α-catenin (present at approximately half the quantity of the other proteins). Doubling the quantity of α-catenin did not result in any discernible association of SHP2 peptide. The
thrombin-elicited dissociation of SHP2 from the cadherin-catenin complex was not due to changes in the quantity of β-catenin associated with VE-cadherin, as evidenced by the co-precipitation study shown in Fig. 4.

We then examined whether there was a change in the SHP2 SH2 binding sites on β-catenin. Extracts from HUVEC treated without or with thrombin were incubated with the SHP2-GST fusion peptide conjugated to agarose. No differences in quantities of β-catenin precipitated with the SHP2-GST fusion peptide were detected in extracts from untreated and thrombin-treated HUVEC monolayers (Fig. 5). Thrombin also stimulated the increased tyrosine phosphorylation of PECAM-1 (data not shown). In contrast to β-catenin, this correlated with greater association of PECAM-1 with the SHP2-GST fusion peptide (Fig. 5). The same pattern of SHP2 binding by β-catenin and PECAM-1 was obtained when the paired extracts from untreated and thrombin-treated HUVEC were analyzed across a range of protein concentrations (25–200 μg) or with ligand-binding blots (data not shown). These results suggested that SHP2 dissociation from the VE-cadherin complex was due to thrombin-mediated modifications of SHP2, not β-catenin.

SHP2 isolated from thrombin-treated HUVEC exhibited a decreased relative mobility when compared with untreated HUVEC (Figs. 2 and 4). Others have demonstrated that growth factor-stimulated changes in the apparent molecular weight of SHP2 are due to increased tyrosine phosphorylation of Tyr246 and Tyr744 near the SHP2 carboxyl terminus (31). These two tyrosines also appear to be important binding sites for proteins with SH2 binding domains such as Grb2 (32). We immunoprecipitated SHP2 from HUVEC extracts that had been boiled in 1% SDS to dissociate SHP2 complexed with proteins. Tyrosine phosphorylation of the immunoprecipitated SHP2 was detected with the antiphosphotyrosine antibody 4G10. Thrombin treatment of HUVEC caused a significant increase in the tyrosine phosphorylation of SHP2 (Fig. 6A) that correlated with SHP2 dissociation from cadherin complexes (Figs. 2, 4, and 6B). When SHP2 was immunoprecipitated from HUVEC pretreated with the Src family kinase inhibitor PP1 (33), negligible tyrosine phosphorylation of SHP2 was detected in extracts from untreated or thrombin-treated cells (Fig. 6A). When SHP2 tyrosine phosphorylation by a Src family kinase(s) was ablated by PP1, thrombin was unable to elicit SHP2 dissociation from VE-cadherin complexes (Fig. 6B), consistent with our hypothesis that thrombin-stimulated modifications of SHP2 promote SHP2 dissociation from the VE-cadherin complex.

This study is the first to demonstrate SHP2 association with a cadherin complex. We have shown that in HUVEC with well...
established cell-cell junctions, a population of the tyrosine phosphatase SHP2 is found associated with VE-cadherin complexes. Upon exposure of HUVEC to thrombin, much of the SHP2 associated with the VE-cadherin complexes becomes tyrosine-phosphorylated by a Src family kinase and dissociates. This provides a mechanism for thrombin-mediated regulation of the tyrosine phosphorylation of β-catenin, γ-catenin, and p120-catenin. The loss of SHP2 from VE-cadherin complexes correlates with increased tyrosine phosphorylation of β-catenin and γ-catenin. Such changes in β-catenin and γ-catenin phosphorylation have been shown to alter their interaction with α-catenin (11, 34), thereby diminishing the cytoskeletal association of VE-cadherin complexes. This would promote the cell-cell junction disassembly and intercellular gap formation detected in endothelial cell monolayers after thrombin treatment and the resulting increased monolayer permeability.

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