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Role of FAK in S1P-Regulated Endothelial Permeability

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Abstract

The vascular endothelium serves as a semi-selective barrier between the circulating contents of the blood and the tissues through which they flow. Disruption of this barrier results in significant organ dysfunction during devastating inflammatory syndromes such as sepsis and acute lung injury (ALI). Sphingosine 1-phosphate (S1P) is an endogenous lipid regulator of endothelial permeability that produces potent barrier enhancement via actin and junctional protein rearrangement and resultant cytoskeletal changes. A key effector protein in this S1P response is focal adhesion kinase (FAK), a highly conserved cytoplasmic tyrosine kinase involved in the engagement of integrins and assembly of focal adhesions (FA) through the catalysis of multiple downstream signals. After stimulation by S1P, endothelial FAK undergoes specific tyrosine phosphorylation that results in activation of the kinase and dynamic interactions with other effector molecules to improve the endothelial barrier. FAK participates in peripheral actin cytoskeletal rearrangement as well as cell-matrix (FA) and cell-cell (adherens junction) junctional complex strengthening that combine to decrease vascular permeability. This review summarizes the current knowledge of the role of FAK in mediating enhanced endothelial barrier function by S1P.

Keywords

FAK; S1P; focal adhesions; endothelium; vascular permeability

INTRODUCTION

The vascular endothelium, composed of a single layer of endothelial cells (EC) and the underlying extracellular matrix (ECM), performs a unique role in regulation of a variety of processes such as vascular tone, hemostasis, angiogenesis and tissue fluid balance. The vascular endothelium of the lung establishes the critical semi-permeable barrier between the vascular, interstitial and alveolar spaces across which exchange of water and solutes occurs (Dudek and Garcia 2001; Komarova and Malik 2010). While both paracellular and transcellular pathways participate in this exchange, the paracellular route, via gap formations, is generally considered to be the primary mode of fluid and inflammatory cell transit. The actin-based endothelial cytoskeleton and a host of actin-binding proteins have been shown to play a key role in this process through generation, linking and balancing of

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opposing forces. Specifically, contractile, centripetal tension forces and tethering cell-cell and cell-matrix forces are thought to modulate cell shape and the resultant gaps between individual endothelial cells (Dudek and Garcia 2001). Imbalance of these forces and, particularly, an increase in the movement of fluid, solutes and inflammatory cells from the vasculature into alveolar airspaces are the hallmarks of devastating inflammatory conditions such as acute lung injury (ALI), and acute respiratory distress syndrome (ARDS) (Wheeler and Bernard 2007). Regulation of this process is an area of intense research. A large volume of work has now identified sphingosine 1-phosphate (S1P) as a potent endogenous regulator of EC permeability that exerts its effects via actin cytoskeletal and junctional protein rearrangement (Wang and Dudek 2009). This review will focus specifically on the role of the integral focal adhesion (FA) protein, focal adhesion kinase (FAK), in the S1P response and enhanced barrier function.

S1P IN ENDOTHELIAL BARRIER FUNCTION

S1P Biochemistry and Membrane Signaling

Sphingosine 1-phosphate (S1P) is a biologically active, angiogenic phospholipid that robustly increases EC barrier function (Wang and Dudek 2009). Multiple studies over the past decade have demonstrated its potent barrier-enhancing effects both in vitro (Garcia, Liu et al. 2001; Dudek, Jacobson et al. 2004; Tauseef, Kini et al. 2008; Zhang, Xu et al. 2010) and in vivo (McVerry, Peng et al. 2004; Peng, Hassoun et al. 2004; Camerer, Regard et al. 2009; Sammani, Moreno-Vinasco et al. 2010). A principal pathway in the production of S1P in most cell types is the breakdown of the structural membrane component, sphingomyelin, which is degraded to ceramide through sphingomyelinases. Ceramide is then deacylated by ceramidase to generate sphingosine, which subsequently is phosphorylated by sphingosine kinases to S1P. This reversible phosphorylation step, as well as irreversible degradation to phosphoethanolamine and hexadecanal by S1P lyase, serves to regulate S1P levels (Hait, Oskeritzian et al. 2006; Tani, Ito et al. 2007). Within the circulation, the majority of S1P is stored within platelets and erythrocytes that serve as repositories of plasma S1P through differential expression of regulatory enzymes (Ito, Anada et al. 2007). When activated, these cells release S1P into the plasma (Yatomi, Ruan et al. 1995; Camerer, Regard et al. 2009) where much of it is bound to circulating proteins like HDL (Argraves, Gazzolo et al. 2008) and its physiologic concentration ranges from approximately 0.3-1.1 μM (Venkataraman, Thangada et al. 2006; Hammad, Pierce et al. 2010).

S1P exerts biological effects through both intracellular and extracellular mechanisms. Its extracellular effects are mediated by five G-protein coupled receptors (S1PR1-5) that bind S1P with high affinity and are expressed to varying degrees in many cell types (Rosen, Gonzalez-Cabrera et al. 2009). Vascular EC primarily express S1PR1-3. These receptors serve as the earliest signal transducers in S1P-induced cytoskeletal rearrangement and subsequent barrier regulation. S1PR1 is closely associated with G_i in a pertussis toxin-sensitive manner and is the primary barrier-enhancing receptor (Garcia, Liu et al. 2001; Dudek, Camp et al. 2007; Sammani, Moreno-Vinasco et al. 2010). In contrast, S1PR2 and S1PR3 are coupled to additional G-proteins with S1P concentration-dependent responses that are primarily barrier disruptive (Singleton, Dudek et al. 2006; Lee, Gordon et al. 2009; Sammani, Moreno-Vinasco et al. 2010). Transactivation of these receptors by other agonists has been described, with barrier promoting agents activating S1PR1 and barrier disrupting agents activating S1PR3 (Finigan, Dudek et al. 2005; Singleton, Dudek et al. 2006; Singleton, Chatchavalvanich et al. 2009). Intracellularly, S1P functions as an important second messenger involved in multiple cellular processes (Strub, Maceyka et al. 2010). Because extracellular S1P can induce intracellular S1P generation through a sphingosine intermediary (Zhao, Kalari et al. 2007), the overall effects of S1P stimulation on EC likely reflect an integration of these extracellular and intracellular processes.

S1P ligation of its receptors rapidly recruits multiple signaling molecules and cytoskeletal effectors into membrane-associated lipid rafts, or caveolin-enriched microdomains (CEM) (Singleton, Dudek et al. 2005). These signaling platforms are essential for mediating S1P effects as their disruption completely abolishes barrier enhancement in pulmonary EC (Singleton, Dudek et al. 2005). CEM participate in this process by bringing into close proximity multiple proteins necessary for transducing S1P receptor ligation into downstream cytoskeletal changes and subsequent improved barrier function (Figure 1). Signaling molecules rapidly recruited (within 5-10 minutes) into CEM by S1P include S1PR1 and S1PR3, PI3 kinase, the Rac GEF Tiam1, c-Abl tyrosine kinase, FAK, and cytoskeletal proteins cortactin, nonmuscle myosin light chain kinase (nmMLCK), α-actinin1/4, and filamin (Singleton, Dudek et al. 2005; Zhao, Singleton et al. 2009; Dudek, Chiang et al. 2010). Multiple tyrosine phosphorylation events occur after S1P within these CEM (Zhao, Singleton et al. 2009; Dudek, Chiang et al. 2010), providing further evidence that these membrane domains are sites of important signaling events after S1P.

Cytoskeletal Rearrangement by S1P

Signaling events initiated by S1P at the pulmonary EC membrane are rapidly transduced by downstream effectors into cytoskeletal changes that determine barrier function. The actin cytoskeleton plays a primary role in this process. Barrier enhancement by S1P is abolished by the actin depolymerizing agent, cytochalasin B, and the actin polymerization inhibitor, latrunculin, but not by the microtubule disruptor, nocodazole (Garcia, Liu et al. 2001). The distribution of actin and myosin is of critical importance to endothelial permeability and barrier function. For example, in most endothelial cell types after exposure to the barrier disruptive agent thrombin, actin forms long transcellular filaments termed stress fibers. These fibers exert a centripetal force leading to overall cell contraction and interendothelial gap formation through the action of non-muscle myosin light chain kinase (nmMLCK) and ratcheting of the actin-myosin fiber complex (Dudek and Garcia 2001). However, this response does not universally increase permeability in all cell types (Troyanovsky, Alvarez et al. 2008). In contrast, during barrier enhancement by S1P, rapid rearrangement of polymerized actin occurs at the EC periphery in association with phosphorylated myosin light chains (MLC) and nmMLCK (Garcia, Liu et al. 2001; Dudek, Jacobson et al. 2004; Brown, Adyshev et al. 2010). Further characterization of how these cytoskeletal changes are translated into biophysical properties has been provided by atomic force microscopy (AFM) analysis of human lung EC. Thrombin stimulation elevates the elastic modulus, an indicator of underlying structural force, within the central region of the cell, while S1P significantly increases the elastic modulus at the EC periphery (Arce, Whitlock et al. 2008). This differential pattern of EC elastic force distribution has been observed with several other barrier-enhancing vs. barrier-disrupting agonists (Birukova, Arce et al. 2009) and demonstrates how F-actin rearrangements correlate with the biophysical properties that regulate permeability.

The Rho family of small GTPases, Rho, Rac and Cdc42, provide a link between membrane-associated receptors like the S1P receptors and downstream mediators that rearrange the cell cytoskeleton (Figure 1). Generally, activation of Rho leads to stress fiber, focal adhesion (FA) formation, contractile, centripetal force generation and increased paracellular gap formation (Wojciak-Stothard and Ridley 2002). In contrast, Rac is associated with generation of focal contacts and lamellipodia that serve to decrease intercellular gaps and lead to enhanced barrier function (Wojciak-Stothard and Ridley 2002). Formation of the cortical actin ring after S1P is mediated by the small GTPase Rac and its downstream effector PAK (Garcia, Liu et al. 2001). S1P-induced Rac activity also recruits the actin-binding protein, cortactin, to the EC periphery (Dudek, Jacobson et al. 2004).

Cortactin is a multifunctional protein well-suited to regulate peripheral actin structure as it binds the Arp2/3 complex and related proteins to stimulate actin polymerization, directly binds F-actin to stabilize filaments, and binds multiple cytoskeletal and effector proteins via its C-terminal SH3 domain, including nmMLCK (Dudek, Jacobson et al. 2004; Ammer and Weed 2008). Experimental data support an important role for cortactin in mediating EC barrier enhancement by S1P as downregulation of its expression significantly attenuates the ability of S1P to increase barrier function in pulmonary EC (Dudek, Jacobson et al. 2004; Zhao, Singleton et al. 2009) and alters the S1P elastic modulus pattern (as measured by AFM) to one resembling unstimulated EC (Arce, Whitlock et al. 2008). Both cortactin and nmMLCK are co-localized to the periphery of the cell and specifically to areas of active membrane ruffling and lamellipodia within 5 min of S1P exposure (Dudek, Jacobson et al. 2004; Brown, Adyshev et al. 2010), and S1P rapidly recruits cortactin and nmMLCK into CEM at the EC periphery in association with c-Abl tyrosine kinase (Zhao, Singleton et al. 2009). Reduction of c-Abl expression by siRNA significantly attenuates S1P-mediated increases in cortical actin formation, EC elastic modulus, nmMLCK and cortactin tyrosine phosphorylation, and barrier enhancement (Dudek, Chiang et al. 2010). These observations demonstrate that c-Abl-cortactin-nmMLCK interaction is functionally important for the peripheral actin cytoskeletal rearrangements that contribute to EC barrier enhancement by S1P (Figure 1).

Junctional Complexes and S1P

Points of contact between individual EC as well as the extracellular matrix (ECM) are mediated by groups of highly specialized proteins. The interendothelial junctions, composed primarily of adherens junctions (AJ) and tight junctions (TJ), mediate the connection between adjacent EC (Vandenbroucke, Mehta et al. 2008). AJs are composed principally of transmembrane vascular endothelial (VE) cadherin linked intracellularly to the catenin complex of proteins, which subsequently connect AJs to the underlying actin cytoskeleton (Vestweber, Winderlich et al. 2009). Disruption of VE-cadherin complexes results in significantly increased cultured EC and lung permeability (Corada, Mariotti et al. 1999; Venkiteswaran, Xiao et al. 2002). TJs can contain several transmembrane proteins including occludin and claudins, which are connected internally to the EC cytoskeleton via a complex of proteins that include zona occludins (ZO) 1-3 (Bazzoni 2006). EC are anchored to the underlying ECM through transmembrane integrins connected intracellularly to focal adhesion (FA) complexes, which consist of multiple proteins including paxillin, talin, vinculin, α-actinin, and FAK (Wu 2005). These FA structures are vital to the formation and maintenance of endothelial monolayers by attaching the cells to their underlying substrate and providing bi-directional signaling between the ECM and EC cytoskeleton (Romer, Birukov et al. 2006). S1P regulates all of these junctional complexes to improve EC barrier function (Figure 1).

S1P enhances AJ assembly in human EC by rapidly recruiting VE-cadherin and the intracellular AJ linker protein β-catenin to peripheral cell–cell contact regions (Lee, Thangada et al. 1999; Sun, Shikata et al. 2009). Recent biomechanical experiments reveal that S1P increases AJ size through induction of Rac activity and myosin-driven force (Liu, Tan et al. 2010). However, the functional role of AJ complexes in the S1P response is complex and incompletely characterized as data from human umbilical vein EC suggest that VE-cadherin expression is not required for the initiation of barrier enhancement but may participate in sustaining improved function over several hours (Xu, Waters et al. 2007). In contrast, downregulation of the AJ linker protein β-catenin significantly attenuates all phases of S1P-induced barrier enhancement in human pulmonary artery EC (Sun, Shikata et al. 2009; Wang, Chiang et al. 2011). S1P also induces the formation of TJ complexes in EC, with downregulation of the intracellular linker protein ZO-1 resulting in decreased barrier

promotion by S1P (Lee, Zeng et al. 2006). Finally, S1P significantly alters FA complexes (Shikata, Birukov et al. 2003; Shikata, Birukov et al. 2003; Wang and Dudek 2009), and FAK plays a central role in this process as will be described in detail below.

FAK IN S1P RESPONSE

Focal Adhesions and Focal Adhesion Kinase

FAs serve to link the intracellular scaffolding and dynamic cytoskeletal rearrangements of cells to the underlying extracellular matrix through transmembrane integrin connections. Integrins exist as multiple α and β glycoprotein chains that noncovalently link in parallel arrays to form over 20 different heterodimers (Romer, Birukov et al. 2006). The distinct dimers formed from these subunits specify the ECM binding target such as collagen, laminin or fibronectin. Intracellularly, integrins interact with the cytoskeleton directly or through linker proteins, which include paxillin, talin, vinculin and α-actinin that together make up the FA complex (Romer, Birukov et al. 2006). Vascular EC express multiple forms of α and β subunits, and these integrins are vital to the formation and maintenance of endothelial monolayers as their disruption leads to cell detachment and a dramatic movement of water and large solutes across the EC layer (Wu 2005; Alghisi, Ponsonnet et al. 2009). Furthermore, multiple lines of evidence suggest the importance of FAs in two directional signaling between the ECM and EC actin cytoskeleton. This signaling function is key in response to mechanical stress, inflammation and barrier regulation (Romer, Birukov et al. 2006). FAs play integral roles in transmitting force generation and consequential changes in cell shape throughout the EC actin cytoskeleton. For example, treatment of EC with the barrier disruptive compound thrombin results in re-organization of FAs into dense foci at the ends of actin stress fibers and subsequent transmission of force to the ECM through the FA complex (Shikata, Birukov et al. 2003; Wu 2005).

Focal adhesion kinase (FAK) is a highly conserved cytoplasmic tyrosine kinase (97% protein sequence homology between human and mouse) involved in the engagement of integrins and assembly of FA through the catalysis of multiple downstream signals (Schaller 2010). This critical enzyme is embryonically lethal when knocked out in mice and participates in remodeling of the cytoskeleton and junctional complexes during multiple fundamental cell processes including angiogenesis, adhesion, and migration (Ilic, Furuta et al. 1995; Schaller 2010). Its structure and function have been reviewed in multiple recent publications (Orr and Murphy-Ullrich 2004; Tomar and Schlaepfer 2009; Frame, Patel et al. 2010; Schaller 2010). Four principal regions compose the molecular structure of FAK (Figure 2). These are an N-terminal four-point-one, ezrin, radixin, moesin binding (FERM) domain, a central catalytic tyrosine kinase domain, a C-terminal focal-adhesion targeting (FAT) domain, and a proline-rich region located between the kinase and FAT domains (Schaller 2010). The FERM domain functions as an autoinhibitory site by interacting with the kinase domain when FAK is inactive, as well as a site for interactions with other molecules that control FAK signaling (Frame, Patel et al. 2010). The FAT domain and its neighboring proline rich region provide additional sites for FAK binding partners, including paxillin and members of the Rho and GAP family of proteins which are important in the localization of FAK and its downstream signaling (Schaller 2010).

This structure of FAK, specifically the FERM domain, plays a key inhibitory role on multiple levels. The FERM domain serves as an auto-inhibitor of FAK activity by directly binding the catalytic domain (Frame, Patel et al. 2010). Furthermore, FERM blocks the site of Src phosphorylation on the activation loop of FAK, and the FERM F1 subdomain prevents autophosphorylation at site Y397, which is important for the recruitment of Src (Frame, Patel et al. 2010). Release of inhibition and subsequent FAK activation appears to require a number of step-wise events. First, the interaction between the FERM domain and

kinase region must be interrupted by the binding of a FERM binding partner, which are numerous and include cytoskeletal related proteins such as actin-related protein 3 (Arp3), ezrin, and the β-integrins (Frame, Patel et al. 2010). Next, Y397 on FAK becomes auto-phosphorylated which allows for further exposure of the FAK activation loop. Finally, Src binding to Y397 and its subsequent phosphorylation of Y576 on the activation loop completes FAK activation. Once phosphorylated, the activation loop in the kinase domain blocks further binding by the FERM domain (Frame, Patel et al. 2010).

FAK and Endothelial Permeability

Multiple studies have demonstrated an important role for FAK in modulating endothelial barrier function, with barrier-promoting effects being described in several models. EC isolated from the embryos of FAK knockout mice exhibit increased permeability compared with wild type EC (Zhao, Peng et al. 2010). In cultured rat lung microvascular EC, expression of a kinase-deficient FAK mutant attenuates hyperosmolarity-induced increases in barrier function and associated peripheral E-cadherin enhancement (Quadri, Bhattacharjee et al. 2003). Similar studies in these EC demonstrate that this dominant negative FAK mutant also inhibits AJ resealing and barrier recovery following H_2O_2-induced permeability (Quadri and Bhattacharya 2007). In human pulmonary artery EC (HPAEC), reduction in FAK expression results in prolonged barrier disruption after thrombin (Mehta, Tiruppathi et al. 2002). Subsequent mechanistic studies in HPAEC have revealed that FAK promotes barrier recovery after thrombin in part by phosphorylating p190RhoGAP to inhibit RhoA activity (Holinstat, Knezevic et al. 2006). Moreover, thrombin activates FAK via a mechanism involving Gβγ and Fyn tyrosine kinase to associate with AJ complexes and stimulate their reassembly following barrier disruption (Knezevic, Tauseef et al. 2009). In addition, tyrosine phosphorylation of FAK in HPAEC is associated with barrier enhancement by HGF and OxPAPC (Birukova, Chatchavalvanich et al. 2007; Birukova, Cokic et al. 2009). However, FAK phosphorylation and increased activity also occur in association with endothelial barrier-disrupting stimuli such as TGFβ (Lee, Kayyali et al. 2007), PMNs (Guo, Wu et al. 2005), and VEGF (Wu, Guo et al. 2003). Thus, FAK has the ability to modulate EC barrier function both positively and negatively depending upon the stimulus involved.

FAK Phosphorylation after S1P

Data accumulated over the past decade indicate that FAK is an integral component of the cell machinery involved in regulation of endothelial barrier function by S1P. FAK expression is necessary for optimal barrier enhancement after S1P as its downregulation by siRNA significantly inhibits maximal (by ~35%) and sustained elevation in HPAEC (Zhao, Singleton et al. 2009). S1P rapidly induces tyrosine phosphorylation of EC FAK within minutes (Lee, Lee et al. 2000; Miura, Yatomi et al. 2000; Shikata, Birukov et al. 2003; Shikata, Birukov et al. 2003), which demonstrates that increased FAK activity occurs in temporal association with S1P-induced barrier enhancement. Studies in HUVEC reveal that FAK tyrosine phosphorylation after S1P is dependent upon G_i signaling, phospholipase C activity, and increased intracellular calcium levels (Lee, Lee et al. 2000). S1P appears to selectively induce phosphorylation at the Y576 site located in the activation loop of the catalytic domain that serves to enhance catalytic activity (Figure 2) (Shikata, Birukov et al. 2003). This pattern contrasts with that of the barrier-disrupting agent thrombin, which induces EC FAK phosphorylation at Y397, Y576, and Y925 (Shikata, Birukov et al. 2003). As noted above, Y397 is an autophosphorylation site that when phosphorylated allows for Src binding and exposure of the FAK activation loop, while Y925 is another Src target that creates a docking site for the Grb2 SH2 domain on FAK, providing a link between FAK and activation of the MAP kinase pathway (Schlaepfer, Hanks et al. 1994; Schlaepfer and Hunter 1996). Because these tyrosine sites regulate FAK binding and activity, the

differential FAK phosphorylation patterns produced by S1P and thrombin are likely to result in agonist-specific FAK interactions with effector proteins and FAK targets that contribute to the diametrically opposed effects of these stimuli on EC barrier function.

Src activity appears necessary for S1P to increase FAK Y576 phosphorylation because the pharmacologic inhibitor PP2 blocks this event while also attenuating S1P-induced translocation of FA proteins to the EC periphery (see below) (Shikata, Birukov et al. 2003). However, PP2 fails to inhibit EC barrier enhancement after S1P (Garcia, Liu et al. 2001), so either other tyrosine kinases involved in the S1P response can substitute for Src, such as c-Abl (Dudek, Chiang et al. 2010), or these events are not required for S1P to improve EC barrier function. Studies of EC barrier enhancement by the S1P analog, FTY720, further demonstrate the complex and poorly understood role of FAK phosphorylation. FTY720, a synthesized compound with strong structural similarity to S1P that has been developed as a pharmaceutical agent for its immunosuppressive effects on lymphocytes (Brinkmann, Billich et al. 2010), specifically induces FAK Y576 phosphorylation (but not Y397) during the timeframe of maximal EC barrier elevation (30-60 min). However, similar to the S1P response, PP2 eliminates this FTY720-induced FAK phosphorylation without affecting the barrier response (Wang, Chiang et al. 2011). Moreover, FAK siRNA has no effect on the peak level of barrier enhancement after FTY720 but does significantly attenuate the magnitude during the sustained phase (> 4 hours) (Wang, Chiang et al. 2011). Interestingly, FAK depletion also has the greatest inhibitory effect on the S1P response during this sustained phase of barrier elevation (Zhao, Singleton et al. 2009). Thus, while the role of FAK expression/phosphorylation remains unclear in the early S1P/FTY720 response, it appears necessary for prolonged barrier enhancement generated by these agonists.

FAK in Cytoskeletal Rearrangement by S1P

As outlined above, extracellular S1P initiates a series of rapid signaling events at the pulmonary EC membrane that are transduced by downstream effectors into cytoskeletal changes that determine barrier function (Figure 1). FAK participates in these S1P signaling events at multiple steps, including being recruited along with other effector molecules into membrane-associated lipid rafts, or caveolin-enriched microdomains (CEM), where it undergoes Y576 phosphorylation and subsequent activation (Zhao, Singleton et al. 2009). These CEM signaling platforms are essential for S1P to increase EC barrier function (Singleton, Dudek et al. 2005). Although the precise targets for FAK activity in these membrane domains have not been identified, S1P stimulation recruits to CEM over 20 barrier-regulatory proteins that exhibit increased levels of tyrosine phosphorylation (Zhao, Singleton et al. 2009), so it appears likely that FAK participates in some of these important signaling events.

In pulmonary EC, S1P stimulates rapid cortical actin rearrangement and lamellipodia formation that is dependent upon activation of Rac GTPase and necessary for optimal barrier enhancement (Garcia, Liu et al. 2001; Dudek, Camp et al. 2007). Given that FAK inhibition or genetic absence results in abnormal lamellipodia formation and movements in other cell types (Tilghman, Slack-Davis et al. 2005; Owen, Pixley et al. 2007), it is likely that FAK plays a vital role in determining EC lamellipodia structure and movement after S1P that leads to improved barrier function. S1P stimulates FAK translocation to the vicinity of these sites of F-actin rearrangement at the EC periphery, in contrast to its distribution under baseline or thrombin-stimulated conditions (Figure 3) (Shikata, Birukov et al. 2003). At the EC periphery, FAK may help regulate Rac activity and lamellipodia formation through multiple mechanisms. First, FAK activation leads to phosphorylation of the scaffolding protein, p130Cas, which can subsequently facilitate Rac activation (Chan, Cortesio et al. 2009; Tomar and Schlaepfer 2009). Secondly, FAK can phosphorylate the adapter proteins β-pix and PKL/GIT2 to form a complex that recruits and activates Rac (Chang, Lemmon et

al. 2007; Yu, Deakin et al. 2009). However, FAK regulation of Rac activation remains incompletely understood as it can interact with multiple Rho GTPase family GEFs and GAPs (Schaller 2010), and its absence has been associated with significantly increased Rac activity in some cell types (Owen, Pixley et al. 2007). Therefore, it is possible that FAK may help regulate Rac activation both positively and negatively after S1P to coordinate F-actin rearrangements to produce EC barrier enhancement. Similarly, FAK directs multiple Rho GEFs and GAPs (Schaller 2010) to regulate Rho activity and cell contraction, which also appear to be necessary for maximal EC barrier enhancement after S1P (Garcia, Liu et al. 2001).

Finally, FAK may participate in S1P-induced actin rearrangement through its interactions with the Arp2/3 complex of actin polymerizing proteins. When FAK is not phosphorylated on Y397, its FERM domain can bind directly to Arp3 in the Arp2/3 complex to enhance Arp2/3-dependent actin polymerization and control protrusive lamellipodia formation and cell spreading (Serrels, Serrels et al. 2007). Arp3 is released once FAK undergoes phosphorylation at Y397 and its kinase becomes activated, which provides a mechanism by which FAK spatially can target Arp2/3-driven actin polymerization at the cell periphery (Serrels, Serrels et al. 2007). Moreover, FAK has the ability to bind and phosphorylate N-WASP, an activator of the Arp2/3 complex (Wu, Suetsugu et al. 2004). FAK-catalyzed phosphorylation of N-WASP regulates its cellular localization and function to provide an additional pathway through which FAK can regulate peripheral F-actin rearrangements after S1P.

FAK in FA and AJ Re-distribution after S1P

EC barrier enhancement by S1P is accompanied by rearrangement of cell-cell (AJ and TJ) and cell-matrix (FA) junctional complexes (Figure 1) (Wang and Dudek 2009). FAK is an integral component in determining FA complex organization and resultant EC barrier function (Wu 2005). A large body of literature has identified FAK as being essential for FA disassembly and turnover in at the leading edge in migrating cells (Ilic, Furuta et al. 1995; Webb, Donais et al. 2004; Tomar and Schlaepfer 2009). For example, quantitative analyses in fibroblasts demonstrate that FAK−/− cells exhibit impaired adhesion turnover and protrusive activity at the cell front (Webb, Donais et al. 2004). Recent evidence suggests that FAK is likely to participate in similar FA-related processes to help improve EC barrier function after S1P. In HUVEC, S1P activates integrin $\alpha_v\beta_3$ in lamellipodia via S1PR1 and induces $\alpha_v\beta_3$ association with FAK and the cytoskeletal protein α-actinin (Wang, Lee et al. 2008), thus identifying FAK as a link between FA structures and cytoskeletal α-actinin, whose expression is required for S1P-induced cortical F-actin rearrangement and EC barrier enhancement (Singleton, Dudek et al. 2005). In addition, immunofluorescent studies have demonstrated that S1P induces the redistribution of both FAK (Figure 3) and the critical FA protein paxillin to the periphery in HPAEC during the time frame of maximal barrier enhancement (Shikata, Birukov et al. 2003; Shikata, Birukov et al. 2003).

Immunoprecipitation experiments provide additional insights into the possible functional effects of FAK redistribution after S1P. Under basal conditions in HPAEC, FAK associates with paxillin at FA, while other pools of these two proteins are distributed in the cytosol (Shikata, Birukov et al. 2003). S1P induces the disassembly of these FA coincident with transient association of FAK with G protein-coupled receptor kinase interacting protein 1 (GIT1) (Shikata, Birukov et al. 2003), which is one of the major ADP-ribosylation factor GTPase activation factors by which Rac and Rho exert influence on FA dynamics (Turner, West et al. 2001). This observation suggests that disassembly of the FA complex is a key component of the cellular effects of S1P stimulation. Cytosolic FAK is then translocated to the EC periphery in association with the PKL/GIT2-paxillin complex, where these proteins may help link newly formed FA with the increased cortical actin ring observed after S1P

(Shikata, Birukov et al. 2003). The time frame of these FA protein rearrangements, which appear to require Src tyrosine kinase activity, correlates well with S1P-induced actin cytoskeletal changes and EC barrier enhancement (Shikata, Birukov et al. 2003; Shikata, Birukov et al. 2003). Another potential mechanism by which FAK-paxillin interaction may contribute to the reorganization of nascent focal contacts after S1P involves the displacement of the dynein-associated protein Nudel from paxillin, which serves to weaken these cell-matrix linkages and increase their turnover (Shan, Yu et al. 2009).

Interestingly, studies of EC barrier enhancement by the pharmaceutical S1P analog, FTY720, demonstrate increased FA complex formation in temporal association with maximal EC barrier enhancement (30-60 min); however, the functional consequences of these complexes are unclear as expression of neither paxillin nor vinculin are required for FTY720 to increase EC barrier function (Wang, Chiang et al. 2011). Furthermore, as noted above and in contrast to S1P, FAK siRNA does not affect the peak level of barrier enhancement after FTY720 but does significantly attenuate the sustained phase (> 4 hrs) (Zhao, Singleton et al. 2009; Wang, Chiang et al. 2011). Therefore, although the functional importance of FA proteins in mediating the permeability effects of S1P and FTY720 remains incompletely characterized, available data suggest they participate in the sustained phase of endothelial barrier enhancement.

FAK expression and activity have been associated with both promotion and disassembly of cadherin-based cell-cell junctions in various models (Avizienyte, Wyke et al. 2002; Yano, Mazaki et al. 2004; Playford, Vadali et al. 2008). Recent data suggest that FAK modulates these cell-cell AJ complexes during EC barrier enhancement by S1P. First, S1P significantly strengthens the association between FAK, VE-cadherin, and the AJ intracellular linker protein β-catenin in HPAEC (Sun, Shikata et al. 2009). Moreover, paxillin also associates with these FAK-VE-cadherin-catenin complexes after S1P, suggesting a dynamic interaction between AJ and FA proteins during EC barrier enhancement. Furthermore, β-catenin is necessary for this interaction as siRNA depletion of β-catenin results in loss of S1P-mediated VE-cadherin association with FAK and paxillin and significantly impairs barrier enhancement (Sun, Shikata et al. 2009; Wang, Chiang et al. 2011). However, as discussed above, the functional role of AJ complexes in the S1P response remains incompletely characterized as VE-cadherin expression is not required in HUVEC for the initiation of barrier enhancement but does participate in the sustained phase over several hours (Xu, Waters et al. 2007). Similarly, the S1P analog FTY720 does not induce the redistribution of AJ proteins during the period of maximal barrier enhancement (30-60 min), nor does silencing the expression of β-catenin inhibit its barrier-promoting effects (Wang, Chiang et al. 2011). However, FTY720 induces cortical redistribution of AJ proteins in association with FAK after 3 hours in human microvascular EC, which again suggests a role in the sustained phase of this barrier enhancing response. Overall, these combined data strongly support the emerging theme that FAK is a critical linking protein between the force generating actin cytoskeleton and cell-cell and cell-matrix junctional complexes that translate S1P signals into structural changes to mediate EC barrier enhancement (Figure 4).

Areas for Future Study of FAK in the S1P Endothelial Barrier Response

FAK protein expression is necessary for maximal and sustained EC barrier enhancement after S1P (Zhao, Singleton et al. 2009), but the mechanism(s) by which it contributes to this permeability effect are not fully characterized. In addition to its tyrosine kinase activity, FAK also functions as an important structural molecule through its interactions with other cytoskeletal and effector proteins via its FERM and FAT domains (Tomar and Schlaepfer 2009; Frame, Patel et al. 2010; Schaller 2010). A recent study using an EC-specific FAK kinase-defective mutant knockin demonstrates that FAK has differential kinase-independent and -dependent functions in vascular development (Zhao, Peng et al. 2010). It is likely that

FAK has kinase—dependent and –independent functions in S1P-mediated EC barrier enhancement, but these roles have not yet been fully elucidated.

In terms of FAK tyrosine kinase activity, the essential targets for FAK phosphorylation after S1P, as well as the precise timing and subcellular localization of these phosphorylation events, remain poorly characterized. Because S1P stimulation recruits over 20 barrier-regulatory proteins to CEM that subsequently undergo tyrosine phosphorylation in these membrane signaling platforms, including FAK (Zhao, Singleton et al. 2009), it seems likely that FAK targets some of these cytoskeletal and signaling molecules. Identification of these targets and characterization of their subsequent functions will improve our understanding of how S1P increases EC barrier function. Another limitation of the current state of our knowledge regarding FAK in S1P barrier enhancement is that the majority of the data have been generated using large vessel conduit EC rather than microvascular endothelium. Given the well-described phenotypic differences between pulmonary EC derived these vascular beds, it is important for future work to better define the role of FAK in the S1P response of microvascular EC.

As a protein with numerous domains and complicated functions, research into FAK is constantly opening new doors of discovery and speculation. For example, recent intriguing developments include the identification of nuclear export signals (NES) and nuclear localization signals (NLS) within the structure of FAK, as well as evidence for the ability of FAK to regulate gene expression in the nucleus (Golubovskaya, Finch et al. 2005; Lim, Chen et al. 2008; Ossovskaya, Lim et al. 2008). To date there is no evidence to suggest that FAK regulation of gene expression participates in the S1P barrier response; however, it is interesting to speculate that such a mechanism conceivably could play a role in the sustained phase of EC barrier enhancement that lasts for many hours. Regardless of whether FAK regulates gene expression to promote EC barrier function after S1P, its profound effects on regulatory phosphorylation, actin cytoskeletal structure and function, and junctional complex disassembly and re-distribution all combine to make a compelling argument for an essential role of FAK in the endothelial barrier response to S1P (Figure 4). Improved understanding of the activation and function of this critical enzyme may lead to novel insights into the regulation of EC permeability and potentially new therapeutic targets to reverse the vascular leak associated with devastating clinical syndromes such as sepsis and ARDS.

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Highlights

- S1P is an endogenous phospholipid that potently enhances the endothelial barrier
- Cytoskeletal and junctional complex rearrangement mediate the S1P response
- S1P stimulates FAK phosphorylation and activation in endothelial cells
- FAK is integral to cytoskeletal and junctional complex rearrangement induced by S1P

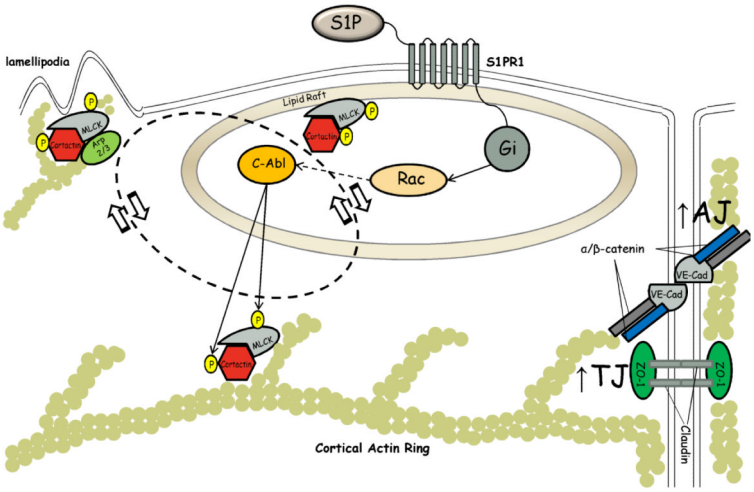

Figure 1. S1P regulates endothelial cell structure to enhance barrier function

Ligation of the S1PR1 Gi protein coupled receptor by S1P rapidly (within 1-5 min) activates Rac and recruits signaling molecules and cytoskeletal effectors such as c-Abl, cortactin, and nmMLCK to lipid rafts (or CEMs). Tyrosine phosphorylation of these molecules is observed both in lipid rafts and at the EC periphery in association with cortical actin and lamellipodia formation. This activated complex likely interacts with Arp 2/3 machinery to produce lamellipodia protrusion at the cell periphery, which serves to increase overlap between adjacent EC. The initiation and precise sequence of events responsible for these protein movements are unclear, but within 5 min after S1P stimulation these proteins are found simultaneously distributed in lipid rafts, cortical actin structures, and peripheral membrane ruffling/lamellipodia (indicated by the bi-directional circle). S1P also induces adherens junction (AJ) and tight junction (TJ) assembly that serve to further strengthening the endothelial barrier. Multiple other signaling and cytoskeletal effector molecules participate in this process as reviewed elsewhere (Wang and Dudek 2009). MLCK, nonmuscle myosin light chain kinase; VE-cad, vascular endothelial cadherin; ZO-1, zona occluden protein-1.

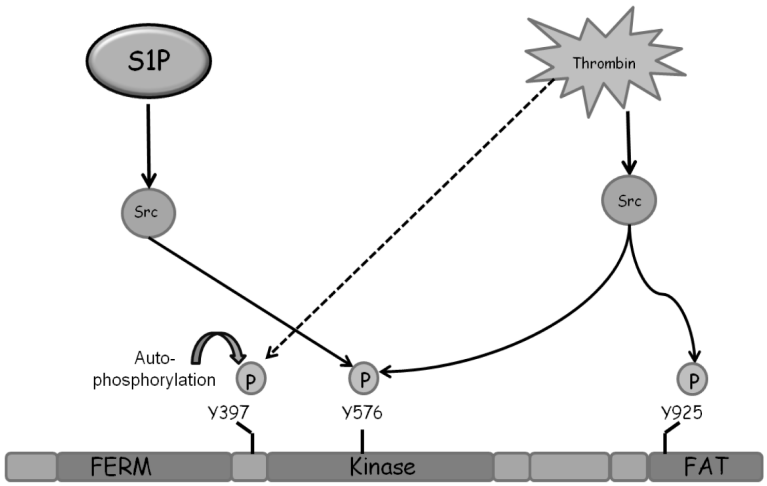

Figure 2. Differential phosphorylation of FAK by S1P and thrombin

Depicted are the principal structural domains of FAK and the location of three critical tyrosine phosphorylation sites [adapted from (Schaller 2010)]. Barrier protective S1P induces the specific phosphorylation of the FAK-activating site tyrosine 576 in a Src-dependent manner. Barrier disruptive thrombin induces Src-dependent phosphorylation of tyrosine 576 and tyrosine 925 as well as auto-phosphorylation of tyrosine 397 by FAK (Shikata, Birukov et al. 2003). FERM, four-point-one, ezrin, radixin, moesin binding domain; FAT, focal adhesion targeting domain; Y, tyrosine residue.

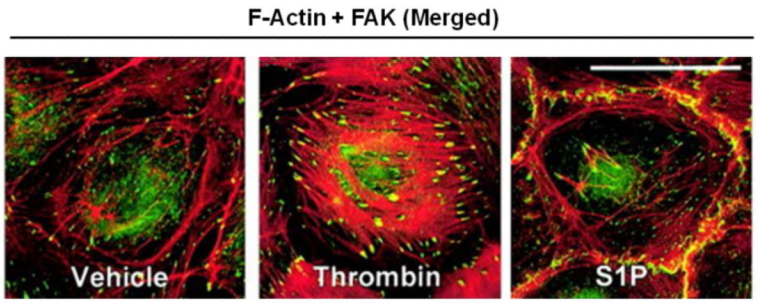

Figure 3. FAK is differentially redistributed by S1P and thrombin

Shown are representative merged images of HPAEC immunostained for F-actin (red) and FAK (green) after stimulation with vehicle, thrombin (100 nM, 10 min), or S1P (0.5 μM, 10 min). Thrombin induces FAK localization to the ends of actin stress fibers (yellow, middle panel) while S1P stimulated cells demonstrate a localization of FAK to the cortical actin ring (yellow, right panel). Bar = 10 μm. Reproduced with permission (Shikata, Birukov et al. 2003).

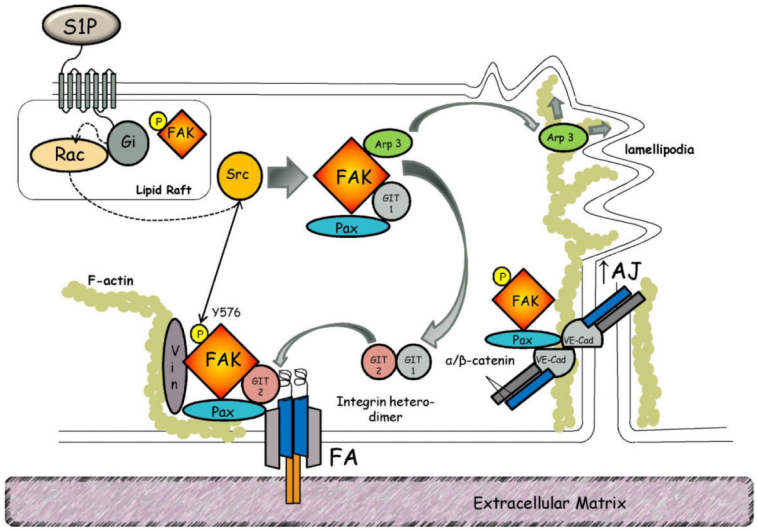

Figure 4. FAK in the S1P barrier-enhancing response

S1P binding to the S1PR1 Gi protein coupled receptor activates Rac and recruits multiple proteins to membraneassociated lipid rafts, including FAK, which exhibits increased tyrosine phosphorylation and presumed activation at this site. Src-dependent phosphorylation of FAK on Y576 also occurs in the cytosol and in existing FA. This phosphorylation releases bound Arp3 from the FAK FERM domain so that it can participate in Arp2/3-catalyzed actin polymerization at peripheral EC membrane ruffling/lamellipodia sites. Src-dependent phosphorylation of FAK on Y576 also transiently increases its association with GIT1 during FA disassembly and rearrangement. FAK, paxillin, and GIT2 then translocate to the EC periphery where they form new focal contacts associated with the increased cortical actin ring produced by S1P. S1P also induces increased peripheral association of FAK, paxillin, VE-cadherin, and β-catenin to strengthen cell-cell AJ linkages. Pax, Paxillin; GIT-1, G protein-coupled receptor kinase interactor-1; GIT-2, G protein-coupled receptor kinase interactor-2 (PKL); Vin, Vinculin; FA, focal adhesion; AJ, adherens junction; VE-cad, Vascular endothelial cadherin.