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Proline-rich region of non-muscle myosin light chain kinase modulates kinase activity and endothelial cytoskeletal dynamics

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ABSTRACT

Disruption of the pulmonary endothelial barrier and subsequent vascular leak is a hallmark of acute lung injury. Dynamic rearrangements in the endothelial cell (EC) peripheral membrane and underlying cytoskeleton are critical determinants of barrier function. The cytoskeletal effector protein non-muscle myosin light chain kinase (nmMLCK) and the actin-binding regulatory protein cortactin are important regulators of the endothelial barrier. In the present study we functionally characterize a proline-rich region of nmMLCK previously identified as the possible site of interaction between nmMLCK and cortactin. A mutant nmMLCK construct deficient in proline residues at the putative sites of cortactin binding (amino acids 973, 976, 1019, 1022) was generated. Co-immunoprecipitation studies in human lung EC transfected with wild-type or mutant nmMLCK demonstrated similar levels of cortactin interaction at baseline and after stimulation with the barrier-enhancing agonist, sphingosine 1-phosphate (S1P). In contrast, binding studies utilizing recombinant nmMLCK fragments containing the wild-type or proline-deficient sequence demonstrated a two-fold increase in cortactin binding ($p < 0.01$) to the mutant construct. Immunofluorescent microscopy revealed an increased stress fiber density in ECs expressing GFP-labeled mutant nmMLCK at baseline ($p = 0.02$) and after thrombin ($p = 0.01$) or S1P ($p = 0.02$) when compared to wild-type. Mutant nmMLCK demonstrated an increase in kinase activity in response to thrombin ($p < 0.01$). Kymographic analysis demonstrated an increased EC membrane retraction distance and velocity ($p < 0.01$) in response to the barrier disrupting agent thrombin in cells expressing the mutant vs. the wild-type nmMLCK construct. These results provide evidence that critical prolines within nmMLCK (amino acids 973, 976, 1019, 1022) regulate cytoskeletal and membrane events associated with pulmonary endothelial barrier function.

Introduction

The acute respiratory distress syndrome (ARDS) is a highly morbid clinical entity marked pathophysiologically by the flooding of the alveolar space with protein rich fluid which leads to severe impairment of gas exchange and hypoxia (Rubenfeld and Herridge, 2007; Ware and Matthay, 2000). Under normal conditions, the pulmonary endothelium forms a thin semipermeable barrier between the vascular space and the interstitial and alveolar spaces allowing for effective gas exchange while preventing the excess leakage of fluid and protein (Dudek and Garcia, 2001). However, this barrier becomes severely compromised during ARDS.

Pulmonary endothelial cell (EC) mechanics play a central role in the regulation of this barrier function as the formation of intercellular gaps and paracellular movement of fluid and solute are the primary mechanisms producing vascular leak in pathologic conditions (Baldwin and Thurston, 2001; Garcia et al., 1995). The combination of EC shape and the formation of tight connections to neighboring cells through junctional proteins determine the degree of intercellular gap formation or closure and are affected by rearrangements of the EC cytoskeleton (Baldwin and Thurston, 2001; Birukova et al., 2009; Dudek and Garcia, 2001). Transcellular contractile forces generated by thick actin filaments termed stress fibers disrupt the EC barrier, while peripherally directed cell–cell and cell–matrix tethering forces seal paracellular gaps and increase barrier function (Dudek and Garcia, 2001). In vitro, the edema generating compound thrombin stimulates the formation of transcellular stress fibers, cell contraction, and rounding to disrupt the EC barrier (Bogatcheva et al., 2002; Garcia et al., 1996). In contrast, physiologic levels of the endogenous phospholipid sphingosine-1-phosphate (S1P) induce actin rearrangement to the cell periphery and formation of a cortical ring which significantly increases barrier function (McVerry and Garcia, 2004; Schaphorst et al., 2003). In addition,

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S1P increases peripheral areas of dynamic actin rearrangement to produce membrane projections, such as lamellipodia, that decrease intercellular gaps and strengthen the EC barrier (Lee et al., 2006).

The actin dynamics that determine barrier function are controlled by a number of cytoskeletal regulatory proteins including cortactin (CTTN) and non-muscle myosin light chain kinase (nmMLCK) (Dudek et al., 2004). Cytoskeletal force generation is mediated by the ATP-dependant ratcheting of actin and myosin bonds catalyzed in EC by nmMLCK, a Ca^{2+}/calmodulin-dependent enzyme (Dudek and Garcia, 2001; Shen et al., 2010). This 1914 amino acid protein is similar to smooth muscle MLCK but with a unique N-terminal region (amino acids 1–923) containing multiple regulatory tyrosine phosphorylation sites for both Src and c-Abl (Dudek et al., 2010) (Garcia et al., 1995; Verin et al., 1998). The C-terminal half of the enzyme contains the catalytic, calmodulin-binding, KRP-binding, and myosin-binding domains (Garcia et al., 1997).

Cortactin is a 546 amino acid protein that participates in multiple aspects of cytoskeletal rearrangement to regulate EC elastic properties and barrier function (Arce et al., 2008; Li et al., 2004). CTTN contains multiple functional domains that regulate actin structure, including a binding site for filamentous actin and an N-terminal acidic domain that stimulates the Arp2/3 complex to polymerize branched actin filaments (Cosen-Binker and Kapus, 2006; Li et al., 2004). CTTN also contains multiple regulatory phosphorylation sites with serine and tyrosine residues located within a proline rich α-helix domain (Zhao et al., 2009). The C-terminus of CTTN consists of a Src homology 3 domain (SH3) that provides a site of interaction for numerous other EC proteins (Cosen-Binker and Kapus, 2006; Zarrinpar et al., 2003). CTTN rapidly translocates to sites of dynamic peripheral actin rearrangement after stimuli that increase EC barrier function (Brown et al., 2010), and inhibition of its expression impairs barrier enhancement by S1P (Lee et al., 2006) (Dudek et al., 2004).

Previous work has demonstrated an association between nmMLCK and CTTN through the latter protein's SH3 domain (Dudek et al., 2002, 2004), and inhibition of this interaction attenuates S1P-induced barrier enhancement (Dudek et al., 2004). Recombinant protein studies revealed no alterations in the enzymatic activity of nmMLCK in response to CTTN binding, but this interaction decreased the ability of nmMLCK to bind F-actin (Dudek et al., 2002). The putative sites of this interaction within nmMLCK were characterized using blocking antibodies and interfering peptides to identify amino acids #972–979 and #1019–1025 as the likely moieties responsible for this binding (Dudek et al., 2002). These sites contain proline rich sequences which conform well to consensus SH3 domain recognition motifs for the CTTN domain (Sparks et al., 1996). The goal of the present study was to characterize the functional effects of critical prolines within the CTTN binding site of nmMLCK to provide additional insights into how these key cytoskeletal proteins regulate EC barrier function.

Materials and methods

Reagents and antibodies

Reagents and chemicals were obtained from Sigma (St. Louis, MO) unless otherwise specified. Cortactin monoclonal antibody-4F11 was purchased from Millipore (Billerica, MA). Anti-Flag monoclonal antibody M5 was purchased from Sigma (St. Louis, MO). Anti-VE-cadherin mouse monoclonal antibody was purchased from Santa Cruz (Santa Cruz, CA). Fluorescent dye labeled reagents and Prolong Gold with DPI were obtained from Molecular probes (Eugene, OR). Cell culture reagents were purchased from Lonza (Walkersville, MD).

DNA construct generation

The wild-type nmMLCK construct was subcloned from the previously described mammalian expression vector pJM1 (Moitra et al., 2008) as Acc65I–EcoRI fragment with N-terminal 6xFlag-tag into pcDNA3.1(+) mammalian expression vector (Life Technologies, Carlsbad, CA). Site directed mutagenesis of this wild-type nmMLCK construct was performed to generate proline to alanine substitutions at amino acid sites 973, 976, 1019 and 1022 by using the QuikChange Multi Site-Directed Mutagenesis Kit (Agilent Technologies, Inc., Santa Clara, CA) according to the manufacturer's protocol (Fig. 1A). Both wild-type and proline deficient mutant nmMLCK constructs were also subcloned into mammalian EGFP tagged expression vector pEGFP-C1 (Life Technologies, Carlsbad, CA). Both wild-type and proline deficient truncated nmMLCK fragments of 206 amino acids from #934 to 1140 containing the putative sites of CTTN binding were generated from the full length constructs by PCR. Primer sequences for truncated human nmMLCK, 5′-CACCAAGA CTGTGTCTGAGGAA-3′ (sense), containing the sequence CACC at the 5′ end of the primer for the directional cloning and 5′-TTAGGTGG TCTTGAGGGTCTTTC-3′ (antisense) were designed by using NCBI Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/primerinfo.html) and subcloned into pENTR™/D-TOPO entry vector by directional TOPO cloning following subcloning by LR recombination to the pDEST™17 bacterial expression vector with N-terminal 6xHIS-tag according to the manufacturer's protocol (Life Technologies, Carlsbad, CA) (Fig. 3A). Rat CTTN in pDsRed-N1 constructs were generously provided previously by Drs. Mark McNiven (MayoClinic) and H. Clive Palfrey (University of Chicago) for use in live cell imaging studies (McNiven et al., 2000).

In silico protein modeling

The online application Protein Homology/analogy Recognition Engine (PHYRE) was used to analyze the 206 amino acid sequences of the recombinant wild type and proline deficient constructs and generate a model of the predicted structure (Kelley and Sternberg, 2009).

Cell culture

Human pulmonary artery ECs (HPAECs) (Lonza, Basel, Switzerland) were cultured in Endothelial Basal Medium (EBM)-2 complete medium with 10% fetal bovine serum (FBS) in a humidified incubator with 5% CO_2 at 37 °C as we have described previously (Dudek et al., 2004; Garcia et al., 2001). Passages 5–9 were used for experimentation. On the day of experimentation medium was changed to serum free media and cells were treated as outlined in specific experimental conditions.

Transfection

ECs were transfected with mutant and wild-type constructs per manufacturer protocol using the X-fect transfection reagent purchased from Clontech (Mountain View, CA).

Co-immunoprecipitation

ECs were grown to confluence in 60 mm dishes as described above. Cells were then treated with either 1 μM S1P or PBS for 10 min. Cells were washed three times with ice-cold PBS and lysed with 0.6 ml per dish of Co-IP lysis buffer (20 mM Tris–HCL; 150 mM Nacl; 1% NP-40; 2 mM EDTA; 0.5 mM PMSF; 0.2 mM sodium orthovanadate; 0.5% Protease and 0.5% phosphatase inhibitor cocktail) (Calbiochem). Cells were scraped and allowed to incubate for 15 min on ice. Cell lysates were passed through a 26 G needle 10 times and centrifuged for 10 min at 15 K rpm at 4 °C. Anti-Flag or anti-cortactin antibody was added (1:120) and incubated for overnight while rotating at 4 °C. The following day, 50 μl of protein G beads (GE Life Sciences, Pittsburgh, PA) was washed three times with Co-IP wash buffer (20 mM Tris–HCL; 150 mM Nacl; 1% NP-40; 2 mM EDTA; 2 mM EGTA; 0.2 mM sodium orthovanadate; 20 mM NaF; 1 mM sodium phosphate). Lysates were then added to beads and rotated at 4 °C for 1.5 h. Beads were