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Quantitative distribution and colocalization of non-muscle myosin light chain kinase isoforms and cortactin in human lung endothelium

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ABSTRACT

Vascular barrier regulation is intimately linked to alterations in the distribution and configuration of the endothelial cell (EC) cytoskeleton in response to angiogenic and edemagenic agonists. Critical actin cytoskeletal rearrangement includes spatially directed increases in myosin light chain (MLC) phosphorylation, catalyzed by Ca^{2+}/calmodulin-dependent non-muscle myosin light chain kinase variants (nmMLCK1- and -2), as well as association of nmMLCK with the actin-binding protein, cortactin. As these associations have proven difficult to quantify in a spatially specific manner, we now describe the utility of intensity correlation image analysis and the intensity correlation quotient (ICQ) to quantify colocalization in fixed and live cell imaging assays in human pulmonary artery EC. From baseline ICQ values averaging 0.216 reflecting colocalization of cortactin–DsRed with EGFP–nmMLCK fusion proteins in resting EC, thrombin-induced EC contraction significantly reduced cortactin–DsRed–EGFP–nmMLCK colocalization (nmMLCK1: ICQ = 0.118; nmMLCK2: ICQ = 0.091) whereas the potent EC barrier-protective agonist, sphingosine 1-phosphate (S1P), significantly increased nmMLCK–cortactin colocalization within lamellipodia (nmMLCK1: ICQ = 0.275; nmMLCK2: ICQ = 0.334). Over-expression of a cortactin–DsRed mutant fusion protein lacking the SH3 domain, known to be essential for cortactin–nmMLCK association, reduced baseline and S1P-mediated live cell colocalization with each nmMLCK variant (nmMLCK1: ICQ = 0.160; nmMLCK2: ICQ = 0.157). Similarly, expression of a truncated EGFP–nmMLCK2 mutant lacking cortactin- and actin-binding domains, markedly reduced basal localization in lamellipodia and abolished colocalization with cortactin–DsRed in lamellipodia after S1P (ICQ = −0.148). These data provide insights into the molecular basis for vascular barrier-regulatory cytoskeletal responses and support the utility of sophisticated imaging analyses and methodological assessment to quantify the critical nmMLCK and cortactin interaction during vascular barrier regulation.
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Introduction

The microvascular endothelium provides a semipermeable barrier that regulates the passage of circulating vascular nutrients and fluids and the extravascular interstitium of all organs. Perturbation in the integrity of this monolayer during tumor angiogenesis or inflammation occurs in response to edemagenic agents such as thrombin, histamine, proinflammatory cytokines and activated leukocytes (Dudek and Garcia, 2001; Garcia et al., 1986; Mehta and Malik, 2006; Petrache et al., 2001) and results in fluid leakage, organ edema and dysfunction. Restoration of endothelial cell (EC) integrity is promoted by natural angiogenic agents such as angiopoietin-1 (Thurston et al., 1999), hepatocyte growth factor (HGF) (Liu et al., 2002; Singleton et al., 2007), and sphingosine-1-phosphate (S1P), a lipid signaling molecule secreted by circulating platelets and erythrocytes (Garcia et al., 2001; McVerry and Garcia, 2005; Schaphorst et al., 2003). Both barrier-disruptive and barrier-restorative processes are now recognized as extensively dependent on dynamic rearrangements of the endothelial cytoskeleton. For example, the potent edemagenic agonist, thrombin, induces formation of thick actin stress fibers that contract EC and disrupt paracellular junctions (Dudek and Garcia, 2001; Garcia et al., 1986), whereas S1P reorganizes the cytoskeleton into dense peripheral bands that restore EC intercellular junctional integrity (Dudek et al., 2004; Garcia et al., 2001; Shikata et al., 2003).

Tensile force generation and contraction of actin filaments in non-muscle cells, such as EC, are mediated by phosphorylation of the regulatory myosin light chain (MLC) subunit on Thr^{18}/Ser^{19}, which catalyzes the ratcheting of actin–myosin bonds resulting in intracellular tension development. This enzymatic reaction involves the multidimensional non-muscle isoform of myosin light chain kinase (nmMLCK), a 1914 amino acid (210 kDa) Ca^{2+}/calmodulin-dependent, actin-binding protein encoded by the *MYLK* gene, which also encodes the 1091 amino acid (108 kDa) smooth muscle isoform as well as the 19 kDa protein known as kinase-related protein (KRP) or telokin (Supplemental Fig. 1A). In addition to smMLCK and KRP, we

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previously identified five splice variants compared to the longest variant (nmMLCK1), with nmMLCK1 and nmMLCK2 being the most abundant isoform variants in many tissues including endothelium (Supplemental Fig. 1A) (Birukov et al., 2001; Lazar and Garcia, 1999).

Thrombin increases nmMLCK activity and results in profound cytoskeletal rearrangement, loss of cortical actin, and rapid and dramatic formation of transcellular stress fibers resulting in increased transendothelial permeability (Dudek and Garcia, 2001). Interestingly, ligation of barrier-enhancing receptors including S1PR1 and c-Met (Dudek et al., 2004; Garcia et al., 2001; Liu et al., 2002) as well as others (Finigan et al., 2005; Singleton et al., 2006), results in recruitment of key signaling molecules and their targets such as p60src, c-Abl, nmMLCK, and the actin- and nmMLCK-binding protein, cortactin, to lipid rafts (Zhao et al., 2009). These molecular interactions result in dynamic activation of nmMLCK and dramatic spatially distinct localization of nmMLCK and nmMLCK binding partners, such as the actin-binding protein, cortactin, within cortical actomyosin rings, events intimately linked to enhanced paracellular junctional integrity and EC barrier enhancement (Dudek et al., 2004; Garcia et al., 2001).

Unfortunately, the inability to quantify nmMLCK association with cortactin in a spatially specific manner has proven to be a major limitation to interrogating the molecular mechanisms underlying cytoskeleton-driven EC barrier regulation. We now report the utility of intensity correlation image analysis and the intensity correlation quotient (ICQ) (Brittain et al., 2009; Li et al., 2004; Racz et al., 2008) to quantify the colocalization of cortactin with nmMLCK1 and -2 isoforms in fixed and live cell assays under conditions of EC barrier enhancement and disruption. Our quantitative results indicate that robust thrombin-induced EC contraction reduces colocalization of cortactin with nmMLCK fusion proteins whereas the potent barrier-protective agonist, S1P, increased colocalization of nmMLCK and cortactin within barrier-enhancing lamellipodia. Our imaging analyses in live cell assays confirm our earlier biochemical studies (Dudek et al., 2002; Dudek et al., 2004) and demonstrate cortactin–nmMLCK association to require the SH3 domain of cortactin (Supplemental Fig. 1B) as well as the cortactin- and actin-binding domains of nmMLCK. Together, these data provide insights into the molecular basis for vascular barrier-regulatory cytoskeletal responses and support the utility of sophisticated imaging analyses and methodological assessment to quantify the critical interaction that occurs between nmMLCK and cortactin during EC barrier regulation.

Materials and methods

Reagents and antibodies

Reagent chemicals, including S1P and thrombin, were obtained from Sigma (St. Louis, MO) unless otherwise specified. Laboratory grade paraformaldehyde was purchased from Fisher Scientific (Fair Lawn, NJ). Cortactin monoclonal antibody 4F11 was purchased from Upstate Biotechnology (Lake Placid, NY). The vector pAcGFP1/Actin was purchased from Clontech (Mountain View, CA) while all other fluorescent dye-labeled reagents and Prolong Gold with DAPI were obtained from Molecular Probes (Eugene, OR). Sterile Dulbecco's phosphate buffered saline (D-PBS) was purchased from Mediatech (Herndon, VA), trypsin was purchased from Invitrogen (Grand Island, NY), and all other cell culture reagents were purchased from Lonza (Walkersville, MD). HMVEC-L transfection reagent was purchased from Amaxa Biosystems (Gaithersburg, MD).

DNA constructs

The nmMLCK constructs used were wild-type nmMLCK1 and -2 and an N-terminal fragment of nmMLCK1 consisting of the first 496 amino acids (Supplemental Fig. 1A). The open reading frames for wild-type nmMLCK1 and -2 were subcloned from the mammalian expression vectors pJM1 and pJM2 (Moitra et al., 2008; Wadgaonkar et al., 2003), respectively, as *Xma*I–*Eco*RI fragments into *BspE*I–*Eco*RI digested pEGFP–C1 vector (Clontech, Mountain View, CA). The resulting plasmids were sequence-verified on both strands and represent the CMV-promoter-driven mammalian expression vectors for EGFP-tagged nmMLCK1 and nmMLCK2. The 496-aa N-terminal fragment of nmMLCK2 was subcloned from the pGEX-6P-3 vector (Amersham Biosciences, Pittsburgh, PA) as an *Eco*RI blunt ended fragment (*Xho*I digestion and fill-in) into an *Eco*RI blunt ended digestion (*BamH*I digestion and fill-in) of pEGFP-C1. The resulting plasmid was verified by sequencing of both cDNA strands and represents the vector for the EGFP-tagged N-terminal fragment of nmMLCK2 (EGFP–nmMLCK2Nterm). Rat cortactin constructs in pDsRed-N1 were generous gifts from Drs. Mark McNiven (Mayo Clinic) and H. Clive Palfrey (University of Chicago) and represented full-length wild-type cortactin and cortactinΔSH3, consisting of amino acids 1–450 (McNiven et al., 2000) (Supplemental Fig. 1B).

Cell culture

Human pulmonary artery endothelial cells (EC) obtained from Lonza (Basel, Switzerland) were cultured in complete growth medium consisting of endothelial growth medium-2 (EGM-2) with 10% fetal bovine serum and incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air as we have previously described (Dudek et al., 2004; Garcia et al., 2001). Endothelial cells were utilized at passages 5–9 and on the day prior to experimentation, cell culture medium was changed to EGM-2/2% FBS.

Transfection

The Amaxa nucleofection system was applied to EC for transient transfection of plasmid DNA (Amaxa Biosystems, Gaithersburg, MD). On the day preceding transfection, EC were seeded at ~70%–80% confluence in complete medium in T-75 flasks (Corning, Lowell, MA). On the day of transfection, cells were washed twice in sterile D-PBS, harvested in 0.05% trypsin, diluted into 10 ml of culture medium, and counted with a hemacytometer. As per manufacturer instructions, 500,000 cells were pelleted at 200 g in 4 °C for 10 min, the supernatant completely aspirated, and cells were resuspended in 100 μL of HMVEC-L kit nucleofection solution. Two micrograms of total plasmid DNA were added to the mixture either as 2 μg of EGFP–nmMLCK construct alone or a combination of 1 μg pEGFP–C1/nmMLCK DNA or pAcGFP1/Actin and 1 μg pDsRed–N1/cortactin DNA. The mixture was immediately transferred to a manufacturer's cuvette, transfected with program S-5 in a Nucleofector™ I machine, followed by the addition of 500 μL of pre-warmed, equilibrated complete culture medium. The mixture was transferred to a 35 mm culture dish containing 1 ml complete medium and a sterile, gelatin-coated 25-mm glass coverslip (for eventual live cell imaging). The cuvette was quickly washed once more with 500 μL of complete medium, which was subsequently added to the same 35 mm culture dish. Transfected cells were allowed to recover in complete medium for ~6–8 h, and then the culture medium was changed to EGM-2/2% FBS and incubated with transfected cells overnight followed by live cell imaging or immunofluorescence staining approximately 24–36 h after nucleofection, a time when peak EGFP–nmMLCK expression has occurred.

Indirect immunofluorescence

Colocalization of the nmMLCK isoform with endogenous cortactin was measured and indirect immunofluorescence (Burkhardt et al., 1997, 1993) of cortactin assayed. EC transfected with one of the three EGFP–nmMLCK constructs were added to a 12-well plate containing a gelatin-coated 18-mm glass coverslip in each well and allowed to recover in complete medium for 6–8 h. Transfected EC were

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