Myoferlin Regulates Vascular Endothelial Growth Factor Receptor-2 Stability and Function

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Myoferlin and dysferlin are members of the ferlin family of membrane proteins. Recent studies have shown that mutation or genetic disruption of myoferlin or dysferlin promotes muscular dystrophy-related phenotypes in mice, which are the result of impaired plasma membrane integrity. However, no biological functions have been ascribed to myoferlin in non-muscle tissues. Herein, using a proteomic analysis of endothelial cell (EC) caveolae/lipid raft microdomains we identified myoferlin in these domains and show that myoferlin is highly expressed in ECs and vascular tissues. The loss of myoferlin results in lack of proliferation, migration, and nitric oxide (NO) release in response to vascular endothelial growth factor (VEGF). Western blotting and surface biotinylation experiments show that loss of myoferlin reduces the expression level and autophosphorylation of VEGF receptor-2 (VEGFR-2) in native ECs. In a reconstituted cell system, transfection of myoferlin increases VEGFR-2 membrane expression and autophosphorylation in response to VEGF. In vivo, VEGF levels and VEGF-induced permeability are impaired in myoferlin-deficient mice. Mechanistically, myoferlin forms a complex with dynamin-2 and VEGFR-2, which prevents CBL-dependent VEGFR-2 polyubiquitination and proteasomal degradation. These data are the first to report novel biological activities for myoferlin and reveal the role of membrane integrity to VEGF signaling.

Membrane repair after injury is characterized by the regulated fusion of subplasmalemmal vesicles with plasma membranes to reseal a damaged area. The molecular machinery responsible for this basic cellular function is beginning to be elucidated, and experiments in Caenorhabditis elegans have discovered that the fusion of intracellular membranous organelles (akin to endomembranes) to the plasma membrane is regulated by the fer-1 gene (1, 2). Dysferlin, otoferlin, and myoferlin are homologs of fer-1 in mammals that were initially identified in skeletal and cardiac muscle cells (dysferlin and myoferlin) or the inner ear (otoferlin) (1, 3). They are characterized by multiple C2 domains that are important for binding to calcium, phospholipids, and phosphotyrosine residues and may be regulated by calcium-dependent, protein-protein interactions. By virtue of their localization in the sarcolemma of skeletal muscle and their calcium-dependent accumulation at sites of membrane repair, ferlins are implicated in various types of muscular dystrophy attributable to defects in vesicle fusion to the plasma membrane (3–6). Mice deficient for dysferlin and myoferlin exhibit strong muscular dystrophy phenotypes (5, 7) and, in the case of dysferlin knock-out, defects in membrane resealing in skeletal muscle fibers. However, the biological and molecular functions of the ferlin family of proteins in mammals outside of skeletal muscle are virtually unexplored.

Recently, by isolating highly purified, caveolae-enriched buoyant membrane microdomains/lipid rafts (CEMs/LRs)4 from endothelial cells (ECs) followed by mass spectrometry-based proteomic analysis, we first reported the unexpected identification of Nogo-B (8). Herein, we report that myoferlin is also highly enriched in CEM/LR microdomains isolated from EC and is highly expressed in blood vessels. RNA silencing combined with functional analysis reveals that myoferlin regulates VEGF receptor-2 (VEGFR-2, also known as Flk-1/KDR) stability and signaling. Moreover, myoferlin-deficient mice

4 The abbreviations used are: CEM/LR, caveolae-enriched buoyant membrane microdomains/lipid raft; MALDI, matrix-assisted laser desorption ionization; EC, endothelial cell; VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor-2; MASMC, mouse aortic smooth muscle cell; HEK, human embryonic kidney cells; BAEC, bovine aortic endothelial cell; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; siRNA, small interference RNA; bMyof, bovine-human myofiber; hMyof, human myofiber; HUVEC, human umbilical vein endothelial cell; MAEC, mouse aortic endothelial cell; PLC, phospholipase C; JNK, c-Jun N-terminal kinase; HA, hemagglutinin; ERK, extracellular signal-regulated kinase; WT, wild type; hNS1, human non-silencing 1.
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(myof−/−) demonstrate reduced VEGFR-2 protein levels and VEGF-mediated vascular hyperpermeability. Taken together, these observations identify myoferlin as a regulator of EC functions and suggest that ferlins in general may be necessary for several plasma membrane events such as signaling in specialized cells.

EXPERIMENTAL PROCEDURES

Cell Culture—EA.hy.926, MASMC, HEK, and BAEC cells were grown in high glucose Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% FBS (HyClone), penicillin-streptomycin (Sigma), and hypoxanthine-aminopterin-thymidine (EA.hy.926 only, Sigma). HUVECs and MAECs were grown in EGM-2 media (Clonetics).

Isolation of CEM/LR and Identification of Myoferlin—CEM/LR preparation from EA.hy.926, BAECs, and HUVECs, and MALDI-time-of-flight mass spectrometry were performed as described (8).

Western Blot Analysis and Immunoprecipitation—The myoferlin antibody was provided by Dr. McNally. We used Phospho-PLC as well as phospho- and total ERK-1/2 (Cell Signaling), phospho- and total JNK (BIOSOURCE and Cell Signaling, respectively). Total PLCy1, VEGFR-2 (Western blot), caveolin-1, EDG1, and B2R were from Santa Cruz Biotechnology (Santa Cruz, CA), whereas HSP90, ubiquitin, and VEGFR-2 (cytometry) antibodies were from BD Biosciences. For pull-down assays, immobilized myoferlin-HA (expressed in HEK cells) was incubated for 2 h with protein extracts solubilized using a β-octoglucopyranoside-based buffer (60 mM, 50 mM Tris-Cl, pH 7.4, 125 mM NaCl) with Complete protease inhibitors (Roche Applied Science) with or without a myoferlin SH3 domain peptide (SIIQGKIPANQLAE). After extensive washing, proteins were blotted using respective antibodies.

Northern Blot Analysis—Northern blot analysis was performed using a PCR-generated probe with the oligonucleotides 5′-GGTCCAGGTCTCCTCCTCCCAATAC-3′ and 5′-TTT- TCTCCTCCAGCCTGTCGCG-3′ and by using the human myoferlin cDNA cloned in pcDNA 3 (Invitrogen). RNA was extracted by using TRIzol (Invitrogen) or mRNAeasy kits (Qia-gen). After pre-hybridization (ExpressHyb, BD Biosciences) at 68 °C overnight, membranes were incubated with [32P]dCTP-labeled probe (Prime-it II kit, Stratagene) for 2 h and washed according to hybridization solution manufacturer’s protocol, proteins were blotted using respective antibodies.

Membrane Repair Assay—This experiment was performed on at least six EA.hy.926 per group in triplicate. EA.hy.926 were used because of their relative bulkiness and high membrane content. Following siRNA treatment (72 h) in a glass bottom Petri dish, cells were incubated in DMEM with FM1–43 (25 μM, Molecular Probes) for 5 min. In the meantime, the Petri plate was mounted in a heated Plexiglas chamber (Zeiss) and incubated at 37 °C, 5% CO2, and 95% humidity. Cells were visualized with an Axiovert microscope (Zeiss), and damage was induced to the cell membrane using an argon laser at full power, and 2300 iterations concentrated on a 5-μm × 5-μm section away from the nucleus. An image was captured before damage, and at every 30 s after injury for a total time of 10 min. For every image taken, the increase in fluorescence was measured by using the Zeiss LSM software as follows. Background for each image was equalized and subtracted (resulting in a pre-damage fluorescence value of “0”) by gradually increasing and equalizing the background intensity around the injury site to observe a variation in fluorescence solely at the site of injury.

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Miles Assay—Myoferlin knock-out (myof−/−) mice were generated as described previously (5). F2 generation myof (+/−) mice were bred to yield (+/+ ) and (+/+) mice used for the following experiments. Under anesthesia, mice (6–10 weeks old) were injected with Evans Blue (30 mg/kg, Sigma-Aldrich). VEGF (500 ng in 25 μl) or saline was injected subcutaneously into the dorsal skin. After 30 min, mice were euthanized, perfused with phosphate-buffered saline and the skin was isolated, the fat was removed with forceps, and skin sections were oven-dried at 55 °C, and weighed. Evans Blue was then extracted from the various skin sections using 250 μl of formamide for 16 h at 55 °C. Evans Blue extravasation was measured spectrophotometrically at 590 nm using a standard curve of Evans Blue in formamide. Total Evans Blue extravasation was expressed as total VEGF-induced extravasation minus saline-induced extravasation.

Immunofluorescence Staining—ECs were grown on glass coverslips, fixed in 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, and treated with antibodies as described (9).

siRNA Treatment—Myoferlin DNA target sequences (Qiagen) were (5′-3′): AACCCTGTCTGGAATGAGATT (bhMyof), AAGGCTGTGTGTCAAAGTITCA (hMyof1), AATGAGG-ACTGTTGTGAAAAATT (hMyof2), and AAGGCCTGTGG-TCAAGTCA for siRNA #2, as well as their respective scrambled controls, AATTCCTCGAGGTGTGAAGT, AACA-TGGTGCACATAGCTTGG, and AACCTTTGTCAGGACAGCATA, with their respective controls AAAAAT-TCAAGGTGTTCCGTG and AATAATGTAAGTCAAGCA.

For cell transfection, ECs were exposed to siRNA complexed with Oligofectamine (Invitrogen) in Opti-Mem-I as described by the manufacturer.

Adenovirus Constructs—Dynamin-2 adenoviruses were generated and used as previously described (10). For 70Z/3-CBL adenovirus, the cDNA (11) was subcloned in the Ad5 shuttle vector and amplified by the University of Iowa gene transfer core facility. Each virus was used at a magnitude of infection of 50.

Proliferation Assays—BAECs or EA.hy.926 cells were seeded in 24-well plates and treated with siRNA sequences for 24 h. Cells were then synchronized for 48 h in DMEM with no FBS. After synchronization, FBS (10%) and VEGF (10−9 M) were added to the cells for an additional 72 h. Cells were then trypsinized and counted using a Z1 counter (Beckman-Coulter).
Apoptosis Assay—We used a caspase-8 activity-based colorimetric assay to determine the level of apoptosis in cells. BAECs were treated for 72 h with siRNA sequences in 100-mm tissue culture plates, and caspase-8 activity was determined according to the manufacturer’s instructions (R&D Systems).

Migration Assay—We used a 48-well modified Boyden chamber assay (Neuroprobe). BAECs were treated with siRNA for 72 h, trypsinized, centrifuged, and resuspended in DMEM with 1% FBS, and migration was performed as described previously (12).

Quantitative PCR—BAECs were treated for 72 h with siRNA sequences, and RNA was extracted with RNeasy columns (QiaGen). Reverse transcriptase reaction was performed on 5 μg of total RNA (Invitrogen), and quantitative PCR was performed on 2 μl by using the Sybr Green reagent (Invitrogen) and the bovine VEGFR-2 (5′-ACACTGACATGGCCTCGCTTTT-3′ and 5′-TCTGGCTGTCCAGAAATTCGTG-3′) and glyceraldehyde-3-phosphate dehydrogenase (5′-TGAGCTCAACGG-GAACTCCT-3′ and 5′-TTGTGAACTGCGAGGACACA-ACCT-3′) primers according to the manufacturer’s protocol. Plates were read on an iCycler quantitative PCR analyzer (Bio-Rad).

Plasmids and Cell Transfection—The human myoferlin cDNA was cloned in pcDNA 3.1 (−) using the NotI and KpnI sites. 3′-End HA-tagged versions of human myoferlin were generated by PCR and verified by sequencing and expression. The human VEGFR-2 cDNA was obtained from Genentech. We used HEK cells (293T) for cell transfection. Cells were seeded in 6-well tissue culture plates, and, after reaching 25% confluency, cells were transfected with 2 μg of control or myoferlin plasmid for 6 h with Opti-MEM I and Lipofectamine 2000. Media was replaced with DMEM with 10% FBS for 16 h. Cells were then retransfected with control plasmids or VEGFR-2 plasmids and grown as described above.

Fluorescence-activated Cell Sorting—HEK cells were trypsinized, rinsed with phosphate-buffered saline, and blocked in phosphate-buffered saline and 1% normal goat serum for 15 min. Cells were then labeled with an anti-VEGFR-2 antibody coupled to phycoerythrin for 1 h (BD Biosciences), washed with phosphate-buffered saline, fixed in 2.5% paraformaldehyde, rinsed, and sorted using a FACSsort flow cytometer (BD Biosciences) set on channel FL2. Data were analyzed using WinMDI 2.8.

Surface Biotinylation—Surface biotinylation of BAECs was performed by using a Sulfo-Link biotinylation kit (Pierce) and the manufacturer’s instructions. Samples were boiled in reducing conditions, and Western blot analyses were performed as described.

NO Release Measurement—BAECs were treated for 24 h with siRNA sequences and were grown to confluency. After a 1-h pretreatment with MG-132 (2.5 × 10−6 M), cells were stimulated for 30 min with VEGF (10−7 M) or ionomycin (5 × 10−6 M) as described.

RESULTS

Identification and Localization of Myoferlin in EC—CEM/LR were isolated by sucrose fractionation from the immortalized human EC line EA.hy.926, proteins were run on a SDS-PAGE gradient gel, and one of the prominent bands characterized by MALDI-reTOF mass spectrometry (8) was myoferlin. Western blot analysis of CEM/LR fractions confirmed the enrichment of myoferlin in EA.hy.926 membranes extracts (lanes 1–3, supplemental Fig. S1A), in freshly isolated (passage 0) human umbilical vein ECs (HUVECs) and bovine aortic endothelial cells (BAECs) (Fig. 1A and supplemental Fig. S1B, respectively). These buoyant membrane fractions were enriched in caveolin-1, the main coat protein of caveolae (lanes 1–6) and lack Golgi/post-Golgi contaminants such as β-coatamer protein (lanes 8–12) as well as markers for bulk plasma membrane such as angiotensin converting enzyme (ACE, Fig. 1A). Cholesterol disruption using methyl-β-cyclodextrin on Triton X-100 protein solubility reveals that at least 25%, 30–55%, and 10–30% of total myoferlin resides in cholesterol-rich, methyl-β-cyclodextrin-sensitive CEM/LR in HUVECs, EA.hy.926, and BAECs.
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A.

B.

C.

D.

E.

F.

FIGURE 2. siRNA-mediated myoferlin knockdown disrupts VEGF-induced proliferation and migration. A, scrambled siRNA (hNS1) and sequences containing three mismatches to its target mRNA (hMyof1) had no effect on myoferlin mRNA levels, whereas homologous bhMyof siRNA attenuated myoferlin mRNA levels in BAECs. B, bovine-human myoferlin siRNA sequences (bhMyof) or specific for human myoferlin (hMyof1) block in a specific and dose-dependent fashion myoferlin protein expression in HUVECs and BAECs. Scrambled control (hNS1) had no effect. β-Coatamer protein was used as a loading control. C and D, siRNA against myoferlin blocked VEGF mitogenic effect (10−8 M) in BAECs and HUVECs without interfering with basal proliferation. Data represent mean ± S.E. (n = 6) repeated two times with similar result. *, p < 0.05 compared with control condition (Day 0 or serum-induced proliferation). E, myoferlin knock-down does not influence apoptosis, measured by caspase-8 activity (n = 9). F, VEGF-stimulated chemotaxis is abrogated in BAECs with disrupted myoferlin expression. *, p < 0.05 compared with control treated cells (n = 9).

respectively (HUVECs shown, supplemental Fig. S1C). Hsp90 and caveolin-1 were used as controls for Triton X-100 solubility and insolubility, respectively. Enrichment of myoferlin in CEM/LR from these cells suggests that myoferlin is a CEM/LR resident protein in ECs.

Next, we examined the expression of myoferlin mRNA in vascular cells. Northern blot analysis using a human cDNA probe (human myoferlin nucleotides 2273–3312) and total RNA isolated from the human endothelial cell line EA.hy.926, HUVECs, mouse aortic ECs (MAECs), mouse aortic smooth muscle cells (MASMCs), and BAEC reveals the expression of myoferlin mRNA (8 kb) in all lines investigated (Fig. 1B).

To examine the subcellular localization of myoferlin in cells, immunofluorescence microscopy was performed. In non-permeabilized COS-7 cells transfected with myoferlin-HA, surface staining of the HA antigen (Fig. 1C) documents the presence of the extracellular C-terminal domain of ferlins (2), and endogenous myoferlin colocalizes with myoferlin-HA in permeabilized COS-7 cells (Fig. 1D). Whole mount immunostaining of endogenous myoferlin in EC lining intact mouse pulmonary arteries reveals that myoferlin (green channel) localizes in puncta throughout the cells and at intercellular junctions. Collectively, these data identify myoferlin as a protein found on the cell surface of cultured EC and is highly localized at the plasma membrane in EC lining intact blood vessels.

EC Deficient in Myoferlin Show Defective Membrane Repair—Because of the importance of dysferlin in mediating membrane repair in damaged skeletal muscle, we studied the importance of myoferlin to membrane resealing following injury to EC using a short interfering RNA (siRNA)-based approach to knock down myoferlin levels. Transfection of primary cultures of BAECs with siRNA duplexes directed against bovine-human myoferlin mRNAs (bhMyof) attenuates myoferlin mRNA expression by up to 92% (Fig. 2A, upper panel) compared with vehicle- and scrambled control siRNA (hNS1). In contrast, transfection of BAECs with an siRNA directed specifically against human myoferlin mRNA (hMyof1, bovine and human RNA display 86% identity at the targeted site) did not result in any reduction in myoferlin expression, confirming both the need for complete homology between the siRNA and the target as well as the specificity of the approach. Both myoferlin siRNAs (bhMyof and hMyof1, 15 and 75 nM) induced dose-dependent decreases in myoferlin expression in HUVECs (Fig. 2B, upper panel) and EA.hy.926 (data not shown) by up to 89% compared with vehicle and hNS1, whereas bhMyof (15 and 75 nM) attenuates myoferlin expression in BAECs by 94% (bottom panel) compared with human-specific myoferlin-, hMyof1-, vehicle-, or hNS1-treated cells (Fig. 2B).

The link between myoferlin and membrane repair assay was investigated by directly assessing resealing efficiency after injury in cultured EC by using a technique similar to that used for quantifying calcium entry following laser injury in ECs (13). Single EA.hy.926 cells displaying normal or deficient myoferlin expression were incubated with FM1–43, a dye that fluoresces only in a lipidic environment such as biological membranes after injury (14), and local membrane disruption was achieved by direct laser irradiation. Following initial membrane injury (time 0), a rapid but weak increase in local membrane fluorescence was observed in control, bhNS-, and hNS1 siRNA-treated ECs, followed by a decrease in local fluorescence, suggesting complete resealing (bhNS1 shown, supplemental Fig. S2, A and B). However, in ECs with attenuated myoferlin expression (bhMyof), a more sustained increase in local fluorescent “patches” was observed (supplemental Fig. S2, A and B) followed by a delayed phase of FM1–43 uptake, thereby revealing incomplete membrane resealing in these cells. These data document that, similar to dysferlin and fer1, myoferlin regulates membrane resealing after physical damage.
VEGF Mitogenic Activity Requires Myoferlin—Because fer-1 is necessary for the motility of spermatozoa in C. elegans (1, 2), basic EC functions, such as proliferation and migration, were examined. Serum-induced proliferation of BAECs and HUVECs (p < 0.05 compared with Day 0) was not affected by pretreatment with non-silencing siRNA (bhNS for BAECs; hNS1–2 for HUVECs) or myoferlin siRNA (bhMyof for BAECs; hMyof1–2 for HUVECs) (48 h, Fig. 2, C and D). In contrast, myoferlin silencing (compared with non-silencing siRNA treatment) reduces agonist-stimulated proliferation (VEGF, 10⁻⁹ M, p < 0.05 compared with serum-induced proliferation, Fig. 2, C and D) an effect not attributable to increased apoptosis (caspase-8 activity, Fig. 2E). Ceramide (50 μM) was used as a positive control.

Because myoferlin silencing inhibits VEGF-induced proliferation, the effects of myoferlin on short term VEGF-mediated biological activity, such as chemotaxis, was assessed. VEGF addition (for 4 h) to the lower chamber of a modified Boyden apparatus induces an increase in BAEC migration treated with vehicle- and bhNS siRNA, and treatment with myoferlin siRNA abrogates VEGF-driven cell migration (Fig. 2F).

Myoferlin Deficiency Disrupts Rapid VEGF-mediated Intracellular Signaling Pathways—Because loss of myoferlin impairs EC mitogenicity and chemotaxis, we examined the effects of myoferlin down-regulation on the VEGF signaling cascade related to proliferation and migration (12, 15), i.e. extracellular signal-regulated kinase-1/2 (ERK-1/2), c-Jun N-terminal kinase (JNK), and phospholipase Cγ (PLCγ), the first enzyme downstream of VEGF receptors (15). VEGF induces time-dependent increases in ERK-1/2, JNK, and PLCγ phosphorylation (Fig. 3A–C, upper panels). Treatment of BAECs with myoferlin siRNA markedly reduced VEGF-mediated changes in phosphorylated ERK-1/2 (Fig. 3A, lower panel) and JNK (Fig. 3B, lower panel) levels compared with vehicle- or hNS1-treated cells, and completely abolished VEGF-induced PLCγ phosphorylation (Fig. 3C, lower panel). Hence, a reduction in myoferlin levels attenuates VEGF-mediated activation of key intracellular signaling cascades.

Myoferlin Deficiency Leads to a Loss of VEGFR-2 Protein Expression—Next, we investigated the expression and auto-phosphorylation pattern of VEGFR-2, the primary tyrosine kinase receptor responsible for a majority of VEGF functions in EC (12). Immunoprecipitation of total VEGFR-2 in control BAECs confirmed previous reports that VEGFR-2 exists as a doublet, with the higher molecular weight form being the predominant species (Fig. 3D, top panel labeled VEGFR-2). Pretreatment of ECs with bhMyof siRNA sequence caused a loss of VEGFR-2 protein (76–84% reduction) compared with vehicle- and hNS1 siRNA-treated cells (Fig. 3D). The effect of myoferlin down-regulation on VEGFR-2 tyrosine phosphorylation was even more robust, inhibiting VEGF-induced phosphorylation of VEGFR-2 by up to 92% compared with vehicle- and hNS1-treated cells (Fig. 3D, p-VEGFR-2). Moreover, two additional siRNA sequences specific for bovine myoferlin (bMyof1 and -2)
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blocked VEGFR-2 expression by 62 and 85% (Fig. 3E, upper panel) compared with their scrambled controls. In HUVECs, two human-specific myoferlin siRNAs (hMyof1–2) attenuated VEGFR-2 expression by up to 71% compared with their scrambled controls (Fig. 3E, lower panel). Direct analysis of BAEC surface proteins by surface cross-linking confirmed that 81% of surface VEGFR-2 is lost following myoferlin knockdown (Fig. 3F). However, myoferlin knockdown did not modulate VEGFR-2 mRNA levels at both 48 and 72 h after siRNA treatment (supplemental Fig. S2C) demonstrating that the link between myoferlin and VEGFR-2 expression is unlikely transcriptionally controlled.

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Myoferlin Modulates VEGFR-2 Stability by Preventing Its Ubiquitination/Degradation—Because the loss of myoferlin decreases the levels of VEGFR-2 protein, we examined the role of myoferlin on VEGFR-2 levels in a reconstituted system to dissect a potential mechanism. In human embryonic kidney (HEK) cells (deficient in VEGFR-2 with low levels of endogenous myoferlin) overexpressing myoferlin-HA, the expression of both VEGFR-2 forms was virtually identical to VEGFR-2 expression in control HEK cells (Fig. 5A). However, the presence of myoferlin-HA markedly increased the levels of VEGFR-2 tyrosine phosphorylation (by 5.2-fold in response to VEGF), and ERK 1/2 phosphorylation compared with cells transfected with VEGFR-2 alone. Because overexpression of myoferlin-HA had a weak effect on overall VEGFR-2 expression levels in HEK cells (that endogenously expressed myoferlin), but greatly enhanced its autophosphorylation state and downstream signaling in response to VEGF, this suggests that myoferlin may influence VEGFR-2 localization at the cell membrane in this reconstituted cell system. Indeed, flow cytometry analysis of the cell surface VEGFR-2 expression in non-permeabilized cells confirmed that myoferlin-HA increased VEGFR-2 surface expression as observed by a rightward shift in fluorescence intensity (Fig. 5B). The observed mean intensity of fluorescence of the cell population expressing VEGFR-2 increased from 51 to 93 in the presence of myoferlin, suggesting that the cells express greater amounts of VEGFR-2 when co-expressed with myoferlin.

We examined the possibility that the decrease in VEGFR-2 levels might be attributable to an increase in its degradation by the ubiquitin/proteasome degradation pathway. Short term pretreatment (6 h) of BAECs with myoferlin siRNA sequences (bhMyof versus scrambled bhNS shown, Fig. 5C) caused a marked decrease (78%) in myoferlin levels 8 h post-treatment but a modest decrease in VEGFR-2 (36%). Concomitantly, myoferlin silencing caused an increase in VEGFR-2 polyubiquitination in VEGFR-2 immunoprecipitates (Fig. 5C), suggesting that the gradual loss of VEGFR-2 after myoferlin gene silencing triggers receptor polyubiquitination, which likely leads to its degradation. This hypothesis was verified by the near-complete rescue of VEGF-induced nitric oxide (NO) release, a well described acute biological activity of VEGF (16, 17), in myoferlin-knock-down cells by the proteasome inhibitor

FIGURE 4. Myoferlin knock-out mice display lower VEGFR-2 expression levels and impaired permeability and angiogenesis in response to VEGF. A, total VEGFR-2 expression in mouse lung extracts in lower in myoferlin-deficient mice. B, vascular permeability assessed by Evans Blue extravasation in un.injected, saline-injected (25 µl) or VEGF protein-injected (40 ng in 25 µl) skin from WT or myof (−/−) mice. Data replicate total Evans Blue extracted formamide in saline or VEGF-injected skin minus total extraction in uninjectected skin. C, quantitative measurement of basal and VEGF-induced Evans Blue extravasation in WT or myof (−/−) mice (n = 6; *, p < 0.05 compared with WT plus VEGF).
MG-132 (Fig. 5D). Ionomycin-induced NO release (a calcium mobilizing agonist that bypasses the receptor-mediated process) is unaffected by myoferlin knock-down, confirming that loss of myoferlin was not exerting a nonspecific effect on the ability of ECs to produce NO. Collectively, these data reveal that myoferlin increases VEGFR-2 protein stability and functional signaling by preventing its ubiquitination and subsequent degradation.

Loss of VEGFR-2 Following Loss of Myoferlin Is Rescued by Dominant-negative CBL and Overexpression of Dynamin-2—Because both ubiquitin ligase CBL (11) and the GTPase dynamin-2 (10) are known to interact with VEGFR-2 and influence its degradation and trafficking, we investigated the role of these proteins on VEGFR-2 expression following myoferlin knock-down in ECs. Infection of control ECs (non-silencing RNA interference treated) with an adenovirus encoding β-gal (lane 1) or dominant-negative form of CBL lacking the RING motif (Ad70Z/3-CBL, lane 2) had no effect on myoferlin or VEGFR-2 expression (Fig. 5E, left). In contrast, infection with Ad70Z/3-CBL rescues the loss of VEGFR-2 expression observed in myoferlin siRNA-treated and Adβ-Gal infected ECs (lanes 4 and 3, respectively). Mechanistically, this implicates CBL as a key enzyme responsible for targeting VEGFR-2 for proteasomal degradation following myoferlin knockdown. In addition, infection of ECs with an adenovirus encoding dominant-negative K44A dynamin-2 reduces VEGFR-2 protein levels as previously reported (lane 6) similarly to cells treated with myoferlin siRNA (Fig. 5E, right). The loss of VEGFR-2 following myoferlin silencing was rescued by 52% with an adenovirus encoding WT dynamin-2 (Fig. 5E, right) suggesting that dynamin-2 activity is required for normal VEGFR-2 levels.

Myoferlin Forms a SH3-dependent Complex with Dynamin-2 and VEGFR-2—Because analysis of the myoferlin domain structure reveals the presence of an SH3 domain that can interact with proline-rich domains, and dynamin-2 contains such a proline-rich domain and forms a complex with VEGFR-2, pulldown experiments were performed to test the hypothesis that a myoferlin-dynamin-2 protein complex is necessary for the proper trafficking of VEGFR-2 and protecting the receptor from CBL-dependent degradation in cells depleted of myoferlin. Pulldown assays reveal that both VEGFR-2 and

FIGURE 5. Myoferlin increases plasma membrane expression of VEGFR-2 by preventing CBL-dependent ubiquitination and degradation. A, co-expression of myoferlin-HA with VEGFR-2 increases VEGFR-2 expression, auto-phosphorylation, and downstream ERK-1/2 phosphorylation following VEGF (10−8 M; 7.5 min) treatment compared with VEGFR-2 transfection alone in reconstituted HEK cells. B, cells were transfected as described in A, and the cell surface expression of VEGFR-2 was analyzed in non-permeabilized cells by flow cytometry. Similar data were found in two additional experiments. C, rapid loss of myoferlin induces VEGFR-2 poly-ubiquitination. D, proteasome inhibitor MG-132 (1-h pretreatment) rescues the loss of VEGF-induced (30 min; 10−8 M) NO release following myoferlin knock-down. Ionomycin (Iono) was used as a control to confirm similar intrinsic ability of BAECs to release NO. *, p < 0.05 compared with hNS1-treated cells (n = at least four individual experiments in triplicate). E, adenovirus expression of dominant negative (70Z/3) ubiquitin ligase CBL or WT dynamin-2 (24 h) rescue loss of VEGFR-2 following myoferlin gene knockdown. All conditions were normalized with β-galactosidase adenovirus. Experiments were performed in triplicate; typical data are shown. F, dynamin-2 and VEGFR-2 form an SH3-dependent complex with myoferlin. Immobilized HA-tagged myoferlin (Myo-HA) was allowed to interact with protein extracts isolated from mouse heart and lungs in a β-octylglucopyranoside-based buffer. To evaluate the role of SH3-dependent interactions, a synthetic myoferlin SH3 domain peptide was added during pulldown experiments. Experiments were performed in triplicate; typical results are shown.
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dynamin-2 (from mouse heart and lung extracts) interact with myoferlin-HA immobilized on an anti-HA resin (Fig. 5F) and not to anti-HA resin alone. Interestingly, addition of a myoferlin SH3 domain peptide completely blocks the dynamin-2 and VEGFR-2 interaction with myoferlin, suggesting an SH3-dependent interaction between myoferlin-HA and the dynamin-2-VEGFR-2 complex. Taken together, these findings indicate that myoferlin complexes with dynamin-2 and VEGFR-2 in an SH3-dependent manner, which most likely prevents CBL-dependent degradation of VEGFR-2.

DISCUSSION

The major finding in this report is the identification of a role of myoferlin in endothelial cells. Consistent with the biological activity of fer1, dysferlin, and otoferlin (1, 2, 7), myoferlin is necessary for membrane resealing after damage, however, myoferlin serves an additional function in VEGF signal transduction by regulating the levels of the receptor, VEGFR-2. We observed that siRNA knockdown of myoferlin in vitro in three different EC lines and the genetic loss of myoferlin in mice in vivo reduce the basal levels of VEGFR-2. Mechanistically, we propose that myoferlin can interact with VEGFR-2 and the GTPase dynamin-2, thus stabilizing VEGFR-2 and preventing its degradation via CBL-mediated ubiquitination. This model is supported by the following data: 1) the loss of myoferlin or expression of dominant negative dynamin-2 reduce VEGFR-2 levels in endothelial cells; 2) co-expression of VEGFR-2 with myoferlin in a heterologous system increases the level of surface VEGFR-2; 3) WT dynamin-2, dominant negative CBL, and pro-tasome inhibition all rescue the loss of VEGFR-2 signaling triggered by myoferlin siRNA, and 4) myoferlin can form a complex with both VEGFR-2 and dynamin-2 via its SH3 domain. These findings rationalize the loss of VEGF mitogenic and chemotactic activities following myoferlin knockdown in vitro, and the loss of vascular permeability in response to VEGF in myoferlin knock-out animals, and suggest a broader role for ferlins in regulating membrane turnover and growth factor signaling.

The detection of myoferlin in CEM/LR is in agreement with a recent study identifying myoferlin at the cell surface of microvascular ECs via in vitro proteomic mapping (18). Moreover, our data on EC membrane resealing support recent evidence describing myoferlin as a likely candidate for muscular dystrophy (3), because myoferlin knock-out mice have defects in skeletal muscle myogenesis and myocyte regeneration. Moreover, the obvious deficiency in VEGF-2 tyrosine kinase signaling observed in myoferlin knockout cells or knock-out mice raises the hypothesis that muscular dystrophy phenotype might be a result of impaired growth factor/tyrosine kinase signaling.

A key finding of the work described herein is the identification of the mechanism by which myoferlin regulates VEGFR-2 expression. VEGF-induced VEGFR-2 autophosphorylation has been shown to promote CBL-dependent ubiquitination, internalization, and degradation in an heterologous cell system (11). Herein, our data show in native ECs the role of CBL in VEGFR-2 signaling and degradation and illustrate the similarity between endogenous VEGFR-2 regulation by CBL in two completely different settings, i.e. following VEGF challenge or myoferlin knockdown.

Another key experiment that has broad implications are data showing an SH3 domain-dependent interaction of dynamin-2 and VEGFR-2 with myoferlin. Analysis of the domains in myoferlin reveals that it contains a putative SH3 domain (3), which may directly bind and form complexes with client proteins containing proline-rich motifs (19), such as dynamin-2. Having the knowledge that dynamin-2 regulates membrane trafficking, forms a complex with VEGFR-2, and prevents its degradation (10), it is likely that CBL-dependent VEGFR-2 degradation is a result of the dissociation of the myoferlin-dynamin-2-VEGFR-2 complex following myoferlin knockdown. This is corroborated by the complete loss of VEGFR-2 induced by K44A dynamin-2 adenovirus and the partial rescue of VEGFR-2 expression in myoferlin knockdown cells by overexpressing WT dynamin-2. In addition, the finding that myoferlin, dynamin-2, and VEGFR2 forms a protein complex links ferlin-dependent membrane resealing to dynamin-2 activity. Membrane resealing is likely an energy-dependent process, but myoferlin and dysferlin, unlike dynamin-2, do not possess ATPase or GTPase activity, which suggests that membrane resealing/fusion may require dynamin-2 GTPase activity (20).

The fact, that dysferlin has recently been shown to be resident in plasma membrane of blood vessels and in caveolae/lipid rafts isolated from human endothelial cells (21), supports our findings about myoferlin in ECs but also suggests that dysferlin cannot fully compensate for the loss of myoferlin. Thus, developmental compensation by dysferlin or other ferlin family members (fer1L4 or fer1L5) can explain the lack of lethal developmental vascular phenotype in mice lacking myoferlin (5). Nonetheless, the discovery of myoferlin and its multiple functions may yield insights into the general mechanisms of membrane turnover and how regulated membrane remodeling may be critical in pathological settings involving aberrant VEGF signaling such as tumor angiogenesis, retinopathy, and vascular diseases.

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