A Novel p38 Mitogen-activated Protein Kinase/Elk-1 Transcription Factor-dependent Molecular Mechanism Underlying Abnormal Endothelial Cell Proliferation in Plexogenic Pulmonary Arterial Hypertension*

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Background: Plexiform lesions comprising proliferative endothelial cells are hallmarks of pulmonary arterial hypertension.

Results: Granzyme B cleaves intersectin-1s and generates a fragment with endothelial cell proliferative potential via phosphorilation of p38MAPK and Elk-1 transcription factor.

Conclusion: Granzyme B cleavage of intersectin-1s and subsequent p38MAPK/Elk-1 activation are critical for endothelial cell proliferation.

Significance: The novel pathogenic p38MAPK/Elk-1 signaling may explain the formation of plexiform lesions.

Plexiform lesions (PLs), the hallmark of plexogenic pulmonary arterial hypertension (PAH), contain phenotypically altered, proliferative endothelial cells (ECs). The molecular mechanism that contributes to EC proliferation and formation of PLs is poorly understood. We now show that a decrease in intersectin-1s (ITSN-1s) expression due to granzyme B (GrB) cleavage during inflammation associated with PAH and the high p38Erk1/2MAPK activity ratio caused by the GrB/ITSN cleavage products lead to EC proliferation and selection of a proliferative/plexiform EC phenotype. We used human pulmonary artery ECs of PAH subjects (ECPAH), paraffin-embedded and frozen human lung tissue, and animal models of PAH in conjunction with microscopy imaging, biochemical, and molecular biology approaches to demonstrate that GrB cleaves ITSN-1s, a prosurvival protein of lung ECs, and generates two biologically active fragments, an N-terminal fragment (GrB-EHITSN) with EC proliferative potential and a C-terminal product with dominant negative effects on Ras/Erk1/2. The proliferative potential of GrB-EHITSN is mediated via sustained phosphorylation of p38MAPK and Elk-1 transcription factor and abolished by chemical inhibition of p38MAPK. Moreover, lung tissue of PAH animal models and human specimens and ECPAH express lower levels of ITSN-1s compared with controls and the GrB-EHITSN cleavage product. Moreover, GrB immunoreactivity is associated with PLs in PAH lungs. The concurrent expression of the two cleavage products results in a high p38/Erk1/2MAPK activity ratio, which is critical for EC proliferation. Our findings identify a novel GrB-EHITSN-dependent pathogenic p38MAPK/Elk-1 signaling pathway involved in the poorly understood process of PL formation in severe PAH.

PAH is a disease of the small pulmonary arteries (PAs) characterized by vascular proliferation, remodeling, and the progressive formation of hallmark PLs that increase pulmonary vascular resistance, ultimately leading to right ventricular failure and death (1). Enhanced proliferation and decreased apoptosis in ECs, pulmonary artery smooth muscle cells, and fibroblasts are central to the pathogenesis of PAH. EC growth and the emergence of phenotypically altered, proliferative ECs in severe PAH are a consequence of initial EC dysfunction, apoptotic death, and subsequent selection of apoptosis-resistant, proliferative vascular cells (2, 3). Human studies of PLs are limited; whether or not they are the cause or the effect of hypertension is not understood. The cellular and molecular mechanisms responsible for PL development are not known. We recently reported that human lung EC dysfunction in a proinflammatory setting is associated with down-regulation of ITSN-1s, a general endocytic protein essential for EC survival, as well as up-regulation of antiapoptotic Bcl-XL and survivin.

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2 The abbreviations used are: PAH, pulmonary arterial hypertension; Ab, antibody; EC, endothelial cell; FD, failed donor; GrB, granzyme B; HIC, immunohistochemistry; ITSN-1s, intersectin-1 short; MCT, monocrotaline; PA, pulmonary artery; PL, plexiform lesion; SRE, serum response element; SH3, Src homology 3; PAEC, pulmonary artery endothelial cell; EH, Eps15 homolog; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; pAb, polyclonal antibody; Ctrl, control.
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s, suggesting that inflammation may not only cause EC dysfunction but also qualitatively change their phenotype. Bcl-XL is increased in animal models of PAH as well as in PAECs isolated from ECPAH (5–7). Significantly, Bcl-XL overexpression renders resistance to GrB-induced apoptosis (8). Evidence indicates that inflammation associated with human PAH attracts CD8+ T-cells, which release the cytotoxic protease GrB (9–12). CD8+ T-cells are significantly increased in the lungs of rats with monocrotaline (MCT)-induced PAH at 3 weeks, suggesting a particular phenomenon related to PAH evolution (13). GrB is implicated in the pathogenesis of several chronic inflammatory diseases mainly because of its well established proapoptotic role (14). Non-cytotoxic roles of GrB, non-apoptotic pathways, and gain of function of the GrB cleavage fragments, still controversial observations, were also reported recently (15).

ITSN-1s is a putative substrate for GrB with a cleavage site at IDQD271GK (16). ITSN-1s is a multimodal protein comprising two Eps15 homology (EH) domains, a central coiled coiled domain, and five consecutive Src homology 3 (SH3A–SH3E) domains (17). This multimodular structure implicates ITSN-1s in multiple protein-protein interactions essential for efficient vesicular trafficking, cytoskeletal rearrangements, regulation of cell signaling pathways, survival, and tumorigenesis (18–23). Thus, ITSN-1s cleavage by GrB, resulting in decreased expression of full-length protein and two cleavage products, may impact essential biological processes and pulmonary EC function. In vivo knockdown of ITSN-1s triggers apoptosis of mouse lung ECs in a process that involves down-regulation of MEK/Erk1/2MAPK survival signaling (22). After only 7 days of ITSN-1s knockdown, the remaining ECs showed phenotypic changes toward increased proliferation and apoptosis resistance, leading to repair and remodeling of the injured lungs; apparently, a signaling switch downstream of Alk5, a broadly expressed TGFβ type 1 receptor (24), from the canonical Smad2/3 to Ras/Erk1/2MAPK signaling protected ECs from impending apoptosis caused by ITSN-1s deficiency and triggered changes in EC phenotype toward enhanced proliferation (22). It has been reported that both TGFβ and bone morphogenetic protein activate Smad-independent MAPK signaling pathways, including Erk1/2 and p38 (25). Phosphorylation of Erk1/2 and/or p38 is increased in experimental models of PAH (26). Although heterozygous mutations in the type II receptor for bone morphogenetic protein underlie the majority of the inherited and familial forms of PAH and may explain the abnormal TGFβ and bone morphogenetic protein signaling, the underlying mechanisms of Erk1/2/p38 activation are not yet understood for a significant group of PAH subjects, and additional factors have been suspected. Thus, we hypothesized that a decrease in full-length ITSN-1s expression due to GrB cleavage during inflammatory reactions associated with PAH and the opposing effects of GrB/ITSN-1s cleavage products on Erk1/2/p38MAPK activation unbalance the activity ratio of p38 to Erk1/2 signaling, leading to EC proliferation and selection of a proliferative/plexiform phenotype.

EXPERIMENTAL PROCEDURES

Materials—Human PAECs were obtained from Lonza (Walkersville, Inc., MD). PAECs isolated from idiopathic PAH subjects were kindly provided by Lerner Research Institute, Cleveland Clinic and Dr. Roberto Machado (University of Illinois at Chicago). X-tremeGENE 9 DNA transfection reagent and In Situ Cell Proliferation kit (BrdU assay) were from Roche Applied Science. ProLong Antifade kit with DAPI was from Molecular Probes (Eugene, OR). MicroBCA (bicinchoninic acid) protein assay reagent, BSA, ECL Western blotting substrate, NE-PER Nuclear and Cytoplasmic Extraction kit, and LightShift Chemiluminescent EMSA kit were from Pierce. Nitrocellulose membranes were from Bio-Rad. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation Assay kit was from ATCC (Manassas, VA). ChIP-IT Express kit and Elk-1 activation kit were from Active Motif (Carlsbad, CA). SB203580 was purchased from Promega (Madison, WI). HyBlot CL autoradiography films were from Denville Scientific Inc. (South Plainfield, NJ). Glutathione-Sepharose 4 Fast Flow beads were from GE Healthcare. Ressimagant heat shock protein (Hsp) 90 was purchased from Enzo Life Sciences (Farmingdale, NY). LPS and GrB were from Sigma-Aldrich, and MCT was from Oakwood Products, Inc. (West Columbia, SC).

Specific antibodies (Abs) were obtained from the following sources. Erk1/2 pAb, phospho-Erk1/2 mAb, p38 pAb, phospho-p38 mAb, JNK pAb, phospho-JNK pAb, PI3K pAb, phospho-PI3K pAb, Akt pAb, phospho-Akt pAb, c-Fos pAb, and myc pAb were obtained from Cell Signaling Technology (Beverly, MA). ITSN-1 Ab against the N terminus of ITSN-1 was generated as described (27). ITSN-1 pAb used for immunohistochemistry (IHC) was from Sigma-Aldrich. Elk-1 pAb, GrB mAb, and PECAM-1 pAb were from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor fluorophore-conjugated antimouse IgG and anti-rabbit IgG were purchased from Molecular Probes. HRP-conjugated reporters were from Cappel, Organon Teknika (Durham, NC).

Cell Culture—Control and transfected ECs were grown in EBM-2 and medium 199 supplemented with 20% FCS as described previously (19). EC_PAH were grown as described (28).

EHITSN (Amino Acids 1–271), Full-length ITSN-1s Constructs, and Transfection Procedure—The cDNA encoding EH domain (amino acids 1–271) of human ITSN-1s cDNA fragment (813 bp) was generated by PCR amplification from ITSN-1L cDNA (a gift from Suzana la Luna, Center for Genomic Regulation, Pompeu Fabra University and Centro de Investigacion Biomedica en Red de Enfermedades Raras, Barcelona, Spain) with High Fidelity PCR enzyme (New England Biolabs) using the following primer pair: ITSN1_F269-EcoRI (5′-AGTAGAATTCCGCCACCATGGCACAGTTTCCAA-3′) and ITSN1_R1082-XbaI (5′-CGTATCTAGAATTCCGCCACCATGGCACAGTTTCCACCCACC-3′) and ITSN1_R1082-XbaI (5′-CGTATCTAGAATTCCGCCACCATGGCACAGTTTCCACCCACC-3′). The PCR products of ITSN-1s (~813 bp) were digested with restriction enzymes (restriction sites are underlined) EcoRI and XbaI (New England Biolabs), purified (Qiagen purification kit), then cloned into expression vector (N-myc) CS-Z9399-M43 (GeneCopoeia) at EcoRI-XbaI sites, and transformed into Escherichia coli.
coli strain Top10 (Invitrogen). The plasmid DNA was extracted from several selected growing clones, and the presence of the ITSN-1s insert was verified with PCR, restriction enzyme (EcoRI and XbaI) digestion, and sequencing confirmation of the integrity of the entire cDNA fragment of ITSN-1s (813 bp of EH domain, which is a 100% match with GenBank™ accession number AF114487) in-frame inside the vector in one of the clones. A similar approach was used for full-length human ITSN-1s cDNA fragment using the following primer pair: ITSN1s_F269-BstBI (5’-AGTATTGGCAACATGGGCTCAGTTTCCAACACCTT-3’) and ITSN1s_R3929-Nhel (5’-CGTACGCTAGCTGCGTGGCTGTTCCACCTGTCG-3’). All transfections were performed using a 3:1 ratio of X-tremeGENE 9 DNA transfection reagent (µl) to DNA (µg) according to the manufacturer’s protocol.

Expression and Purification of GST Fusion Proteins—ITSN(1–440)-GST was generated by subcloning the PCR-amplified ITSN-1, residues 1–440, into the pGEX-4T-1 vector (GE Healthcare). The following primer pair was used: ITSN1_F269 (5’-CCCGGAATTCTACCATGCTCTTCCAACACCTT-3’) and ITSN1_R1588 (5’-CGTACGCGGCGCTTACTCCAGTTTTGCAGCCTCTCG-3’).

For mutagenesis, aspartic acid (D) at position 271 of EH domain of ITSN1 was mutated to glutamic acid (E). PCR primers bearing the mutated sequence, ITSN_D271E_R (5’-CCCTCTCGTGAATTTCCCTTGTGATCAAAGG-3’) and ITSN1_D271E_F (5’-CTTTCTGACATTGAAATCTTCTTCTGG-3’), were used, and the amplified PCR products with the mutation D271E, were digested with EcoRI and NotI and subcloned into the EcoRI-NotI sites of pGEX-4T-1 vector.

For the resulting cDNA vectors were verified by DNA sequencing to ensure sequence integrity, they were transformed into E. coli BL-21(DE3) pLysS (Invitrogen). The GST fusion proteins were then purified as described previously (29).

MCT-induced PAH in the Mouse—PAH was induced in mice as described (30). MCT was dissolved in 0.1 M HCl, neutralized with 0.1 M NaOH to pH 7.4, and sterilized through a 0.22-µm filter. CD1 mice (3 months; 25 g) were injected subcutaneously with MCT at 30 mg/100 g of body weight once a week for 8 weeks. Six mice (three males and three females) were used per experiment. All animals were sacrificed 8 days after the last MCT administration. Lungs (free of blood) were excised and a growth curve was generated to relate the cell number per well. Cell proliferation was determined using the MTT cell proliferation kit in accordance with the manufacturer’s instructions. Briefly, triplicate aliquots of cells (10⁶ cells suspended in 100 µl of complete EC medium) were seeded onto a 96-well plate, and serial dilutions were prepared in EC medium. Cells were cultured for 48 h followed by addition of 10 µl of MTT reagent to each sample. After a 5-h incubation, 100 µl of detergent was added to each well, and the plate was covered and kept in the dark at room temperature overnight. Absorbance was measured at 595 nm in a microtiter plate reader on the following day. Parallel triplicate experiments using non-treated ECs were performed, cells were counted using a hemocytometer, and a growth curve was generated to relate the A₅₇₀ values to the cell number per well.

Cell Proliferation Assay—Cell proliferation was determined using the In Situ Cell Proliferation kit. Cells were grown on coverslips for 48 h and then transfected with myc-GrB-EHₜₕₛₜₐₙ as above. BrdU incorporation was performed according to the manufacturer’s instructions. Briefly, cells were incubated in culture medium containing 10 µM BrdU labeling solution for 6 h at 37 °C. Cells were then washed with PBS and fixed, and the DNA was denatured followed by incubation with BrdU-FLUOS Ab (45 min at 37 °C) in a humid chamber. Cells were again washed with PBS, and the coverslips were mounted using the ProLong Antifade kit. The BrdU-positive cells were counted on high power field images, and data were expressed as the number of BrdU-positive cells per 50 high power fields.
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**Elk-1 Transcription Factor Assay**—Nuclear extracts of control and transfected cells were prepared using the NE-PER Nuclear and Cytoplasmic Extraction kit according to the manufacturer’s protocol. The nuclear extracts were then analyzed by ELISA (TransAM kit with colorimetric readout quantifiable by spectrophotometry) in a 96-well plate containing the immobilized Elk-1 consensus site oligonucleotide. Activated Elk-1 was detected via an Ab against phosphorylated Elk-1 followed by an HRP-conjugated reporter Ab. The plates were read at 450 nm using a Dynex plate reader. Data from triplicate wells in three different experiments were expressed as a mean ± S.E.

**Immunofluorescence Microscopy**—ECs grown on plastic coverslips were washed with cold PBS (three times for 5 min), fixed (methanol for 7 min at −20 °C), quenched (1% BSA in PBS for 1 h at room temperature), and incubated with appropriate primary Ab diluted in 0.1% BSA in PBS for 1 h. After washing with 0.1% BSA in PBS (three times for 10 min), the cells were incubated for 1 h with appropriate reporter Abs, washed again as above, then mounted on glass slides using ProLong Antifade reagent, examined, and photographed in a Zeiss Axioslumer M1 microscope equipped with a digital camera.

IHC on paraffin-embedded human lung tissue sections was performed using ITSN-1 Ab (C-terminal epitope; the only commercially available Ab efficient in IHC); the generally accepted EC marker, CD31; GrB Ab; and phospho-p38 Ab. All were followed by the appropriate Alexa Fluor 488- or Alexa Fluor 594-conjugated reporter as described (22). CD31 and GrB Abs were used at 1:200 dilution in 0.1% BSA in PBS, whereas ITSN-1 and phospho-p38 Abs were used at 1:100 dilution.

**Gel Shift Assay**—The gel shift assay was carried out with the Light Shift chemiluminescent EMSA kit according to the manufacturer’s instructions. Briefly, 20 fmol of biotin-labeled double-stranded probe (Sigma-Aldrich) containing the Elk-1 binding site (underlined) in the c-fos serum response element (SRE; 5′-TCGACAGGATGTCCATATTAGGACATCTGCGTCAGCTCGA-3′) was incubated with 10 μg of nuclear extract in a 20-μl binding reaction containing 2 μl of binding buffer (100 mM Tris, 500 mM KCl, and 10 mM DTT, pH 7.5), 1 μg of poly (dl-dC), 2.5% glycerol, 5 mM MgCl2, and 0.05% Nonidet P-40 for 20 min at room temperature. For competition experiments, a 100-fold excess of unlabelled probe was added to the reaction. The DNA-protein complexes were resolved on a 5% nondenaturing polyacrylamide gel, transferred to a positive nylon membrane (Fischer Scientific), cross-linked in a 1800 UV Stratalinker. Then the membranes were incubated with streptavidin-HRP followed by chemiluminescent substrate before being exposed to HyBlot CL films to visualize the DNA-protein complexes. 2 μg of anti-Sap-1a and anti-Elk-1 Ab was added for the supershift assay.

**ChIP Assay**—The ChIP was performed using a ChIP-IT Express kit according to the manufacturer’s instructions. Briefly, control and myc-GrB-EHITSN-transfected ECs (1.5 × 107 cells; 48 h post-transfection) were fixed in 1% formaldehyde (10 min at room temperature). The cell lysates were centrifuged to pellet the nuclei at 5000 rpm for 10 min at 4 °C. DNA was sheared into 200–800-bp fragments using a Branson sonicator 450 (five pulses at 25% power; each pulse consisted of 20-s sonication followed by a 1-min rest). After centrifugation, an aliquot (10 μl) of the sheared DNA was saved as the input sample for PCR. Sheared chromatin (10 μg) was then incubated with 2 μg of rabbit IgG or Elk-1 Ab and 25 μl of protein G magnetic beads overnight at 4 °C. After reversal of cross-linking and protein digestion with protease K, precipitated DNA and input DNA were purified using the QIAquick PCR purification kit (Qiagen) and subjected to real time PCR amplification with SYBR Green dye on an Applied Biosystems (Carlsbad, CA) real time thermocycler. c-fos promoter fragment containing the Elk-1 binding site was amplified using forward primer 5′-TCCGTACAAGGTTAAAGG-3′ and reverse primer 5′-CTCTCGTGAGCATTTG-3′. An equal volume of cDNA was amplified with Go Taq Green Master Mix (Promega, Madison, WI) in a C1000 thermal cycler (Bio-Rad). The reactions consisted of a 3-min denaturation step at 95 °C, 32 cycles of denaturation at 95 °C for 30 s followed by annealing for 30 s at 60 °C, and extension for 30 s at 72 °C followed by 5 min at 72 °C. The PCR products were resolved on a 1.2% agarose gel and visualized using ethidium bromide staining. Relative amounts of RNA were assessed by comparing the amount of PCR product for ITSN-1s with the PCR product for cyclophilin.

**Human PAH Biospecimens**—ECs were derived from the lungs of three patients with idiopathic PAH at the Cleveland Clinic Foundation (28). IHC studies were performed on 12 PAH and one failed donor lung tissue (FD2_Ctrl) biospecimens (paraffin-embedded lung tissue) identified from autopsy files at Rush University and the University of Chicago. Frozen PAH lung tissue and FD2-Ctrl (kindly provided by Dr. Anna Ryan-Hemmes, Allergy, Pulmonary, and Critical Care Medicine, Van- derbilt University) were used for biochemical investigations. The studies were approved by the Rush Human Subject Committee. Informed consent from the patients was obtained in all cases. Clinical diagnosis, underlying conditions, and other pertinent clinical and laboratory data were reviewed. Lung histological sections were analyzed in each case.

**Statistical Analysis**—All data are expressed as mean ± S.E. (error bars). Statistical analysis was performed using Student’s t test and analysis of variance for comparison of variance between different groups. A value of p < 0.05 was considered significant.

**RESULTS**

**GrB Cleaves ITSN-1s in Vitro and in Vivo**—The identification in ITSN-1s of a potential cleavage site (IDQD27) for GrB prompted us to confirm this cleavage in vitro and in vivo. For in vitro assessment, we generated a truncated human ITSN-1s, tagged with GST (ITSN(1–440)-GST; molecular mass, 78 kDa) as described (29), that contained the GrB cleavage site.
Affinity-purified ITSN(1–440)-GST (Fig. 1A, lane a) was then exposed to recombinant human GrB. Proteolysis of ITSN-1s by GrB generated two cleavage products with \( M_r \) 28,000 and 50,000, consistent with the predicted cleavage site (Fig. 1A, lane b). Site-directed mutagenesis (aspartic acid (Asp\(^{271}\)) mutated to glutamic acid) generated an ITSN(1–440) protein resistant to GrB cleavage and confirmed the specificity of the cleavage (Fig. 1A, lane d versus lane c). Recombinant Hsp90, a known GrB substrate (34), was used as a positive control (Fig. 1A, lane f (arrows) versus lane e). To address the susceptibility of ITSN-1s to native GrB and to evaluate the in vivo cleavage, we exposed mice to LPS for 1, 4, and 6 h. The bacterial endotoxin induces a strong immune response in mice, including an increase in GrB and perforin expression (35, 36). Western blot using an Ab against the N terminus of ITSN-1s (27) applied on LPS-treated mouse lung lysates 4 h post-LPS exposure revealed the loss of full-length ITSN-1s protein expression and presence of the 28-kDa N-terminal fragment, GrB-E\(\text{H}_{\text{ITSN}}\) (Fig. 1A, lane h versus lane g). This is consistent with cleavage of ITSN-1s at the predicted site by GrB produced by the CD8\(^+\) T-lymphocytes in LPS-treated mouse lungs. Moreover, Western blot of LPS-treated mouse lung lysates with GrB Ab indicated the presence of GrB as early as 1 h post-LPS treatment (not shown) and significantly higher levels at 4 h (Fig. 1A, lane i). Cleavage of ITSN-1s by GrB was minimal at 1 h post-LPS treatment, whereas at 6 h post-LPS exposure, the levels of GrB and the extent of ITSN-1s cleavage were similar to those at the 4-h time point (not shown). No GrB immunoreactivity was detected in untreated mice (Fig. 1A, lane k). To further confirm the in vivo cleavage of ITSN-1s by GrB and its connection to PAH, we applied similar analyses on lung tissue of PAH animal models.
ventricular pressure (30). MCT was administered to mice by
subcutaneous injections of 30 mg of MCT/100 g of body weight
for 8 weeks as described previously (30). At the end of the treat-
ment, the lungs were collected, and tissue lysates were prepared
and further subjected to immunoblotting analyses. Decreases
in full-length ITSN-1s and the GrB-EHITSN cleavage product
were easily detected (Fig. 1A, lanes i and j versus lane g), further
confirming the in vivo cleavage of ITSN-1s by GrB in the lungs
of the MCT-treated mouse. GrB immunoreactivity was also
detected in MCT-treated mouse lung lysates (Fig. 1A, lanes i
and j). Weaker immunoreactivity for the GrB-EHITSN fragment
was found in FD1-Ctrl lysates (Fig. 1A, lanes m and n). Similar results were obtained when lung tissue lysates of
an MCT rat model of PAH were analyzed (Fig. 1B). All MCT-
treated rat lung lysates also show immunoreactivity for the
GrB-EHITSN fragment. Actin was used as a loading control.
Given the high endothelial content of lung tissue (37, 38), these
findings are highly significant for ECs of the lung. Thus, we
concluded that ITSN-1s is a substrate of GrB, and its proteolytic
cleavage is associated with the PAH condition in animal
models.

Lung ECs of PAH Subjects Express Low Levels of Full-length
ITSN-1s—Next, expression of ITSN-1s was investigated in
explanted human lung tissue of a 32-year-old white female
transplanted for severe PAH; tissue was sampled from two dif-
f erent locations (PAH I and PAH II). A marked decrease in
ITSN-1s protein and significant immunoreactivity for the GrB-
EHITSN were easily detected by comparison with FD1-Ctrl (Fig.
1C). Weaker immunoreactivity for the GrB-EHITSN fragment
was also detected in the FD1-Ctrl lysates. Given the previous
reports of inflammatory markers, including GrB and perforin,
in bronchoalveolar lavage and alveolar lymphocytes during
acute and chronic lung rejection (39, 40), we investigated GrB
expression in both FD1-Ctrl and PAH lung tissue lysates and
found that both were immunoreactive to GrB (Fig. 1C). How-
ever, GrB immunoreactivity in FD1-Ctrl lysates is 60% lower
than under PAH conditions as estimated by densitometry.
Thus, GrB produced during lung rejection may account for the
GrB-EHITSN presence in the FD1-Ctrl lysates. Actin was used as
a loading control. The observations strongly suggest that the
28-kDa fragment is the result of GrB cleavage of ITSN-1s.

We also evaluated the expression of ITSN-1s protein in
EC_{PAH}. Commercially available human normal PAECs were
used as controls (EC_{Ctrl}). ITSN-1s down-regulation is obvious
in all EC_{PAH} by reference to EC_{Ctrl} (Fig. 1D); however, the
degree of ITSN-1s down-regulation among EC_{PAH} ranged from
30 to 80%. A 28-kDa protein band that was immunoreactive to
ITSN-1 Ab was also detected only when longer ECL exposures
of the nitrocellulose membranes were applied (not shown).
Given the lack of CDB^{+} T-cells and GrB in cultured ECs, we
also evaluated by RT-PCR the levels of ITSN-1s mRNA in
EC_{PAH} by reference to EC_{Ctrl}. EC_{PAH} show on average about
50 lower levels of mRNA (Fig. 1E). Real time PCR as described
(4, 28) confirmed the extent of ITSN-1s mRNA down-regula-
tion in EC_{PAH} compared with EC_{Ctrl} (not shown). These results
strongly indicate a stable EC phenotype and a possible regula-
tion of ITSN-1s and EHITSN expression in late stage PAH at
mRNA levels and by alternative mRNA splicing, a process
highly characteristic to ITSNs (41). Altogether, the findings
demonstrate that ITSN-1s is down-regulated in lung EC_{PAH}.

The level of ITSN-1s in lung ECs of human PAH subjects was
further evaluated by ITSN-1s/CD31 IHC on paraffin-embed-
ded tissue sections. We analyzed 12 PAH cases (Table 1). Rep-
resentative histology (H&E) of lung tissue from PAH specimens
showed PA remodeling, intimal fibrosis, and complex PLs (Fig.
2, a–k). Control lung sections showed no evidence of PA
remodeling or interstitial inflammation (Fig. 2f). For IHC stud-
ies, we used an ITSN-1 Ab against a C-terminal epitope (the
only commercially available Ab efficient for staining of paraffin
embedded-tissue) that does not recognize the N-terminal GrB-
EHITSN. We focused on highly rich EC areas (PL-like) that show
hypercellularity and strong CD31 (accepted EC marker) staining
(Fig. 3, A and B). A complex lesion with focal proliferation of
several endothelial channels and partial destruction of the arte-
rial wall (Fig. 3A, panel a1) shows low ITSN-1s immunoreac-
tivity (panel a2) and limited CD31/ITSN-1s co-localization
(Fig. 3A, panel a3, arrow). The thin walled lymphatic (Fig. 3A,
panels a1 and a4 (asterisk)) in close PL proximity shows barely
detectable ITSN-1s immunoreactivity as well. DAPI was used
for nuclear staining (Fig. 3A, panel a5). Patchy ITSN-1s staining,
most likely associated with airway epithelial cells (Fig. 3, panel a4, arrowheads), was commonly detected. Clusters of
proliferative ECs surrounded by concentric intimal thickening
(Fig. 3B, panels b1 (circled area) and b2) and lacking ITSN-1s
(Fig. 3B, panels b2 and b2.1) are seen in the lumen of a small PA.
Lack of ITSN-1s is characteristic not only of ECs but also of

| Autopsy no. | Etiology | Race      | Sex | Age  | Duration of disease | Cause of death | Pulmonary arterial pressure | Cardiac output | PLs |
|------------|----------|-----------|-----|------|---------------------|----------------|----------------------------|---------------|-----|
| 1          | Idiopathic | Hispanic  | F   | 47   | 7 years             | RV failure     | NA                         | NA            | 12  |
| 2          | Idiopathic | African-American | M   | 51   | 3.5 years           | RV failure     | 53                        | 4             | 6   |
| 3          | Idiopathic | Caucasian | F   | 27   | 2 months            | RV failure     | NA                         | NA            | 5   |
| 4          | Idiopathic | Hispanic  | F   | 29   | 7 years             | RV failure     | 52                        | 4.42          | 8   |
| 5          | Idiopathic | African-American | F   | 53   | 23 years            | Cancer         | 63                        | 6.76          | 1   |
| 6          | Idiopathic | Caucasian | F   | 71   | 18 years            | RV failure     | 59                        | 3.8           | 2   |
| 7          | Idiopathic | Caucasian | F   | 76   | 6 years             | RV failure     | 46                        | 4.6           | 5   |
| 8          | Idiopathic | Caucasian | F   | 48   | 7 years             | RV failure     | 59                        | 3.05          | 7   |
| 9          | Idiopathic | Caucasian | F   | 56   | 6 years             | Upper GI bleed | 54                        | 3.3           | 2   |
| 10         | Idiopathic/familial? | Caucasian | M   | 42   | <7 years            | RV failure     | 69                        | 5.07          | 3   |
| 11         | Lupus     | Filipino  | F   | 45   | 15 years            | RV failure     | 45                        | 0.33          | 3   |
| 12         | Chronic pulmonary embolism | African-American | F   | 64   | 4 years             | RV failure     | 82                        | 4.73          | 3   |

| Disease | Cause of death | Pulmonary arterial pressure | Cardiac output | PLs |
|---------|----------------|-----------------------------|----------------|-----|
| 25706   |                 |                             |                |     |

TABLE 1
Characteristics of autopsied patients

F, female; M, male; NA, not applicable; RV, right ventricle; GI, gastrointestinal.
ITSN-1s staining was also decreased in ECs lining the small blood vessels not yet detect-ably affected by remodeling in PAH specimens (Fig. 3D, inset) compared with control (Fig. 3C, inset). Note also the scarce ITSN-1s staining in all other resident lung cells of the PAH specimen compared with the FD2-Ctrl (Fig. 3D). Weak ITSN-1s staining using an ITSN-1s Ab supposed to recognize not only the full-length protein but also the C-terminal GrB cleavage fragment strongly supports the concept that, in late stage PAH, ITSN-1s expression may also be regulated at the mRNA level as also suggested by the ECPAH phenotype. Altogether, these observations provide undeniable evidence that ITSN-1s defi-ciency is associated with the PAH condition in human.

GrB Immunoreactivity in the Microenvironment of PLs in Severe Human PAH—We next used IHC on paraffin-embed-ded human PAH lung tissue sections to investigate the presence of GrB within PLs and its association with ECs. We examined 57 PLs with three to 12 PLs per section (Table 1). Three of the 12 cases examined showed a lower number of PLs, Cases 6 and 9 (two PLs) and Case 5 (one PL). Based on the emerging clinical and biochemical evidence indicating that GrB can be expressed in other cell types of immune and non-immune origin such as smooth muscle cells (42) or synthesized perhaps by ECs in an autocrine manner in PAH, we evaluated GrB presence by IHC using GrB Ab. GrB immunoreactivity was variable; frequently, prominent GrB staining was associated with the small PAs of PAH lungs, either perivascularly (Fig. 4A) or intraluminally (Fig. 4C), in most of the specimens. GrB was detected either in close proximity of ECs or co-localizing with CD31, used for positive identification of ECs (Fig. 4, A and C, panel c1). DAPI was used for nuclear staining. GrB co-localization or immedi-acy to ECs lining the pulmonary vessels with a diameter greater than 100 μm (medium and large vessels) was not common (Fig. 4B). Even for PAH specimens with less prominent GrB immunoreactivity (Fig. 4D), proximity of ECs of small PAs (Fig. 4D, panel d4) and co-localization with CD31 were detected (Fig. 4D, panels d4 and d4.1). GrB immunoreactivity was not detected on lung sections obtained from the available paraffin-embed-ded FD2-Ctrl (Fig. 4E). Moreover, GrB immunoreactivity was detected in the microenvironment of concentric obliterative lesions frequently in close proximity of ECs (Fig. 4G, arrowheads and insets g1 and g2). H&E staining of the same lesion (Fig. 4F) revealed muscularization of the pulmonary arte-riole in the center, increased adventitial tissue around the ves-sel, and some dilated thin walled vessels (arterioles or venules) at the very edge. A complex PL (Fig. 4H) shows GrB immuno-
ITSN-1s, GrB, and Plexogenic PAH

FIGURE 3. ITSN is down-regulated in human PAH specimens. Lung tissue sections of PAH (A, B, and C) and FD2-Ctrl (D) were subjected to IHC using ITSN Ab and CD31 Ab. Panel a1 illustrates that CD31 immunoreactivity of complex lesion with focal proliferation of several endothelial channels and partial destruction of the arterial wall. ITSN-1s pAb/anti-rabbit IgG-Alexa Fluor 594 staining (panel a2) is barely detected. DAPI staining (panels a3 and b2) of the nuclei documents the hypercellularity and the concentric thickening/distribution of intimal cells. The merged image (panel a4) illustrates co-localization of CD31 with ITSN-1s remnants (arrow) in several ECs of the damaged arterial wall as well as ITSN-1s immunoreactivity associated with pulmonary epithelial cells (arrowheads). ITSN-1s immunoreactivity is barely detected at the level of a thin walled lymphatic in close proximity of the PL (A, panels a1 and a4, asterisk). Clusters of proliferative ECs (B, panel b1, circled area) lacking ITSN-1s (panels b2 and b2.1) surrounded by concentric intimal thickening are seen in the lumen of a small PA. C, inset, ITSN immunoreactivity is associated with ECs lining the blood vessels in FD2-Ctrl. D, inset, low ITSN-1s staining in ECs lining the small blood vessels not yet detectably affected by remodeling in PAH specimen. The results are representative for 12 PAH cases. Scale bars, 30 (A, B, C, and D) and 25 μm (panel b2.1).

reactivity (panel h1) often in close proximity of ECs (panels h2 and h4, arrows). DAPI staining of the nuclei revealed the typical concentric cell proliferation leading to luminal obliteration (Fig. 4H, panel h3). Thus, GrB is present in the milieu of the concentric obliterator lesions and PLs, which are temporally and etiologically related (43). Moreover, GrB can target pulmonary ECs, suggesting that GrB may cleave and down-regulate ITSN-1s protein in PAH lungs.

Myc-GrB-EH1TSN Has EC Proliferative Potential—Previous studies indicated that ITSN-1s or EH domains alone activate mitogenic signaling (20, 22). To address whether the GrB-EH1TSN has similar potential, we cloned it into pcDNA3.1Myc/His vector and expressed it in PAECs as described previously (19). Efficient expression of myc-GrB-EH1TSN protein was detected by Western blot of cell lysates using myc Ab at 48 h post-transfection (Fig. 5A). In addition, immunofluorescent staining of transfected ECs (Fig. 5C, panel c1) further demonstrated efficient protein expression and a subcellular distribution reminiscent of full-length ITSN-1s (19). No myc immunoreactivity was detected in untreated ECs (Fig. 5B). Next, control (Fig. 5D) and myc-GrB-EH1TSN-transfected (Fig. 5E) ECs were subjected to BrdU cell proliferation assay. Morphometric analyses indicated that GrB-EH1TSN expression caused a 40% increase in BrdU-positive cells compared with non-transfected ECs. ECs transfected with myc-tagged full-length ITSN-1s (myc-ITSN) were used for comparison. Only a modest increase (14%) was noted under these conditions (Fig. 5F), consistent with previous reports that within full-length ITSN-1s EH domains are under the inhibitory control of SH3A–E (20). Transfection with the empty vector did not affect the number of BrdU-positive ECs by reference to controls (not shown). Furthermore, we performed the MTT cell growth/proliferation assay to analyze biochemically the proliferation of the whole myc-GrB-EH1TSN-transfected cell population. Cell growth was significantly higher in myc-GrB-EH1TSN-transfected ECs compared with control cells (Fig. 5G). A growth curve was generated to relate the $A_{570}$ values to the cell number per well. The extent of cell growth increase, calculated on three successive points on the curves, indicated a 50% increase in the number of myc-GrB-EH1TSN-expressing ECs compared with controls (Fig. 5H). We concluded that the GrB-EH1TSN cleavage product has EC proliferative potential.

Myc-GrB-EH1TSN Specifically Activates p38MAPK—Because expression of myc-GrB-EH1TSN increases EC proliferation and because ITSN-1s has been implicated in the regulation of Erk1/2MAPK signaling due to its interaction with mSos, a guanine nucleotide exchange factor for Ras (20), we next addressed the effects of myc-GrB-EH1TSN on MAPK activation. Western blot with specific phospho-Erk1/2, JNK, and p38 Abs applied on lysates of control and myc-GrB-EH1TSN-transfected cells indicated that myc-GrB-EH1TSN expression 48 h post-transfection activates p38MAPK and has no effect on JNK (Fig. 6A). Myc-GrB-EH1TSN-expressing cells, however, show decreased Erk1/2MAPK activation, consistent with reports of a negative cross-talk from p38 to Erk1/2 that occurs only in non-transformed cells because p38 can enhance MEK dephosphorylation or up-regulate protein phosphatase 2A (44). Given some limited evidence regarding a role for ITSN-1 in the regulation of the PI3K/AKT/Akt and Erk1/2MAPK pathways, we addressed whether myc-GrB-EH1TSN expression would affect PI3K/AKT/Akt or Erk1/2MAPK activation.
Akt signaling pathway in neurons (45), we also examined a possible role for GrB-EHITSN in PI3K/Akt activity. No detectable changes in the phosphorylation status of these two kinases in ECs expressing the GrB-EHITSN were noted (Fig. 6A).

Time course analysis of p38 activation in myc-GrB-EHITSN-transfected cells indicates strong p38MAPK activation at 48 h post-transfection, the optimal time point for myc-GrB-EHITSN expression; it remains persistent but decreases at 72 and 96 h post-transfection (Fig. 6B, lanes c–e). Myc-ITSN-transfected ECs, used for comparison, showed minimal p38 activation (Fig. 6B, lane b). No p38 phosphorylation was detected under control conditions (Fig. 6B, lane a). To determine whether p38 activation accounts for proliferation of myc-GrB-EHITSN-transfected ECs, ECs were treated with a selective p38MAPK inhibitor, SB203580 (10 μM), and then subjected to BrdU incorporation as above. The proliferative response of myc-GrB-EHITSN-transfected ECs was about 47% inhibited by reference to transfected cells without

FIGURE 4. GrB immunoreactivity in the PAH human lungs and the microenvironment of PLs. Representative GrB/Alexa Fluor 594-CD31/Alexa Fluor 488 IHC of PAH human lung tissue sections illustrates close proximity between ECs and GrB immunoreactivity (A, C, and D); frequently GrB co-localizes with CD31 (C, panel c1) or was detected inside ECs labeled by CD31 (panels d4 and d4.1). B, large PA from PAH specimen does not show GrB staining. E, FD2-Ctrl lung sections do not show detectable GrB immunoreactivity. F, representative H&E staining of a concentric (onion skin) obliterative lesion in human PAH lungs. G, GrB (arrows) within the same obliterative lesion. Arrowheads in G and insets g1 and g2 (boxed areas in G) illustrate GrB immunoreactivity in close proximity of ECs. H, GrB/Alexa Fluor 594-CD31/Alexa Fluor 488 IHC of a large PL shows GrB immunoreactivity (panel h1), the high EC content (panel h2), and frequent presence of GrB in close proximity of CD31-labeled ECs (panel h4, arrows). DAPI staining of the nuclei is shown (panel h3). The results are representative for 12 PAH cases. Three independent IHC experiments were performed. Scale bars, 25 (A, B, and C), 50 (D and E), 10 (panels c1 and d4.1), 40 (F and G), 20 (panels g1 and g2), and 50 μm (H).
SB203580 treatment (Fig. 6C). We concluded that myc-GrB-EH\textsubscript{ITSN} specifically activates p38\textsuperscript{MAPK}, and this activation accounts for its EC proliferative potential.

**Myc-GrB-EH\textsubscript{ITSN} Expression Causes Nuclear Export of p38\textsuperscript{MAPK}**—Morphological evaluation of p38\textsuperscript{MAPK} distribution in myc-GrB-EH\textsubscript{ITSN}-expressing ECs by fluorescence microscopy revealed the presence of unphosphorylated p38\textsuperscript{MAPK} inside the nucleus, in the perinuclear area, and throughout the cytosol in both myc-GrB-EH\textsubscript{ITSN}-transfected ECs and untreated cells (Fig. 6D, panels d1 and d2). However, activated p38\textsuperscript{MAPK} was present predominantly in the cytosol of myc-GrB-EH\textsubscript{ITSN}-expressing ECs (Fig. 6E, panel e1), consistent with previous reports demonstrating that activated p38 undergoes nuclear export and accumulates in the cytosol (46). Additionally, we observed that for some myc-GrB-EH\textsubscript{ITSN}-transfected ECs phospho-p38\textsuperscript{MAPK} staining is brighter, suggesting either more efficient expression of the fragment in these cells or EC heterogeneity and enhanced response to myc-GrB-EH\textsubscript{ITSN} expression. Phospho-p38 staining in control ECs was low/barely detectable (Fig. 6E, panel e1). Nuclear export of p38\textsuperscript{MAPK} and prominent cytosolic phospho-p38 immunoreactivity were also detected in EC\textsubscript{PAH} (Fig. 6E, panel e3); the barely detectable GrB-EH\textsubscript{ITSN} expression by Western blot of EC\textsubscript{PAH} lysates may account for p38 activation in these cells. When paraaffin-embedded lung tissue sections were subjected to phospho-p38 IHC (Fig. 6F), we detected phospho-p38 immunoreactivity within EC profiles labeled by CD31 Ab (Fig. 6F, arrowheads). Co-localization of phospho-p38 with DAPI nuclear staining was not observed, consistent with activation and nuclear export of p38\textsuperscript{MAPK} into the cytosol of pulmonary ECs of PAH specimen. A complex PL comprising proliferative ECs shows phospho-p38 immunoreactivity (Fig. 6G). Given the high EC content, the phospho-p38/CD31 co-localization is significant. Activation of p38 was not detectable in the FD\textsubscript{2}-Ctrl human lung (Fig. 6H).

**Concurrent Expression of GrB/ITSN-1s Cleavage Products Affects p38/Erk1/2 Signaling**—Because under physiological settings ITSN-1s cleavage by GrB generates two cleavage products, the N-terminal fragment with EC proliferative potential via p38 activation and the C-terminal product comprising the five SH3A–E domains with dominant negative effects on Ras/Erk1/2 signaling (21), we next addressed the effects of their concurrent expression on p38/Erk1/2\textsuperscript{MAPK} activity in the MCT mouse model (Fig. 7A). Mouse lung lysates were analyzed by Western blotting with specific phospho-p38 and Erk1/2 Abs followed by densitometry. We detected on average a 4-fold increase in activation of p38 in MCT-treated mice by comparison with untreated mice with a consistent 15% decrease in total p38 kinase expression. In addition, the Erk1/2 phosphorylation in MCT-treated mice was decreased by more than 3-fold compared with controls. Activation of p38 was more prominent in male mice, whereas Erk1/2 inhibition was more obvious in female mice, which are known to be more susceptible to MCT inflammatory effects. Analyses of p38/Erk1/2 activity were also carried out on human lung PAH tissue specimens sampled from two different locations (Fig. 7B). Densitometric analyses revealed a 4.8-fold increase in p38 activation and 4.5-fold...
decrease in Erk1/2 activity. In addition, similar to the MCT mouse model, the decrease in total p38 protein expression was evident; actin confirmed the equal loading. Altogether, the observations are consistent with a role for GrB/ITSN-1s cleavage products in controlling the p38/Erk1/2MAPK signaling activity.

Myc-GrB-EHITSN Activates Elk-1 Transcription Factor and c-fos Immediate Early Response Gene—Because Elk-1, a nuclear transcription factor known to be downstream of p38MAPK, stimulates the expression of immediate early response genes involved in cell growth and proliferation, its activation was evaluated by ELISA. Nuclear extracts of cells transfected with myc-GrB-EHITSN showed ~50% higher Elk-1 activation compared with control and only 20% higher Elk-1 activation in ITSN-transfected ECs (Fig. 8A). Because the primary function of Elk-1 is the regulation of growth-related proteins, mainly c-Fos, we evaluated c-Fos expression by Western blotting of the nuclear extracts (Fig. 8B). Densitometry indicated that cells expressing myc-GrB-EHITSN showed a 4.8-fold increase in c-Fos expression compared with control cells, whereas those overexpressing full-length ITSN-1s showed a 3.2-fold increase, consistent with the recorded Elk-1 activation. Two transcription factors, Elk-1 and Sap-1a, bind efficiently with the serum response factor to the SRE of c-fos promoter to mediate gene expression in response to MAPK activation. To address whether there is any advantage between Elk-1 and Sap-1 in binding the SRE of c-fos promoter, nuclear extracts of control (Fig. 8C, lane b) and GrB-EHITSN-transfected ECs (Fig. 8C, lanes c–g) as well as biotin 3'-end-labeled DNA fragment containing the putative protein binding site from human c-fos gene (lane a) were subjected to a gel shift assay. Shift complexes were seen with the nuclear extracts of transfected ECs (lanes c, d, and f), reflecting the
binding of a nuclear factor to the probe. The binding reaction was performed at room temperature (lane c) and 37 °C (lane d) with no detectable difference in the formation of shift complexes. This binding is specific because it was competed out with 100-fold excess unlabeled oligonucleotide probe (lane e). To determine whether the transcription factor in the shift complex was Elk-1 or Sap-1a, we performed the supershift assay in the presence of Abs to the C terminus of Elk-1 or Sap-1a; neither of them interfered with protein-DNA binding. The Ab to Elk-1 caused a supershift band (lane g), whereas the Ab to Sap-1a caused no supershift (lane f). No shift was detected when control ECs were analyzed (lane b).

To examine whether GrB-EHԭғ cleaves Elk-1 binding to c-fos SRE in vivo, we performed ChIP using Elk-1 Ab. Rabbit IgG was used as a control. The immunoprecipitate was subjected to DNA extraction and PCR to amplify the region encompassing the corresponding Elk-1 binding sites in the human c-fos SRE. GrB-EHԭғ cleaved and significantly increased Elk-1 binding on the c-fos SRE in myc-GrB-EHԭғ-expressing ECs (Fig. 8D). Based on these findings, we concluded that myc-GrB-EHԭғ-mediated p38MAPK activity specifically turns on Elk-1 transcription factor and c-fos gene.

DISCUSSION

This study has demonstrated that the proteolytic cleavage of ITSN-1s by GrB exerts complex effects on growth and proliferation of PAECs that are significant for formation of PLs in severe PAH. We have found that cytotoxic protease GrB cleaves the pro-survival protein ITSN-1s and generates two biologically active products with the N-terminal fragment possessing EC proliferative potential. ITSN-1s cleavage occurs at IDQD271GK, a well conserved sequence among mammals, suggesting that during inflammatory processes associated with increased CD8+ T-cells and GrB expression the ubiquitously expressed ITSN-1s protein is cleaved, and as result, full-length protein expression is decreased. This is significant taking into account that ITSN-1s is a multifunctional protein and that chronic deficiency of ITSN-1s contributes to EC proliferation and vascular remodeling as well as lung malignancies (22, 23). The MCT-induced PAH mouse and rat models were used to demonstrate that the cytotoxic protease GrB cleaves ITSN-1s in vivo, leading to decreased full-length protein expression as well as the presence of the GrB-EHԭғ cleavage product. Although it is recognized that the response to MCT is variable among species, strains, and even animals because of differences in hepatic metabolism by cytochrome P450 (47), the 8-week MCT treatment induces PAH in the mouse model and allows enough time for the development of proliferative arteriopathy (30); rats apparently die of cardiac and renal dysfunction before the formation of occlusive vascular lesions (48). In addition, using human lung PAH specimens, we show that GrB immunoreactivity is associated with the small PAs and PLs and that ITSN-1s is significantly down-regulated compared with FD-Ctrl specimens. These observations are consistent with the idea that during PAH progression when T-cell inflammation is persistent GrB cleaves ITSN-1s and generates the N-terminal cleavage product with EC proliferative potential. This observation seems uncommon taking into account the well documented proapoptotic effects of GrB (14) and that EC is not a typical GrB target. During an inflammatory response, ECs lining the blood vessels are exposed to nonspecifically released GrB and perforin (9). Previous reports demonstrated that the proinflammatory EC dysfunction is associated with ITSN-1s down-regulation and Bcl-XL overexpression (4) and that Bcl-XL is able to suppress apoptotic cell death induced by ITSN-1s down-regulation (27) and GrB/perforin exposure (8). Although non-apoptotic pathways promoted by GrB and gain of function of the GrB cleavage fragments cannot be ruled out...
as a possibility for EC survival, it is very likely that Bcl-XL-mediated suppression of caspase activation induced by GrB (16) occurs during inflammation associated with PAH, and thus ECs are protected against GrB-induced apoptosis.

The C-terminal cleavage product, GrB-SH3A-EITSN, by sequestering mSos has dominant negative effects on Ras/Erk1/2MAPK signaling (21) (Fig. 9). Worth mentioning is that the GrB-SH3A-EITSN can also bind Smad2, which is able to bind SH3 domains of adaptor proteins such as AMSH-2 (associated molecule with the SH3 domain of STAM (signal transducing adaptor molecule); Ref. 49). If this occurs, the interaction may inhibit Smad2-dependent TGFβ signaling and augment EC proliferation. Although the two GrB cleavage products promote both proliferative and apoptotic signaling in lung ECs, the utilization of these signals appear to be cell type- (location of EC) and cell context-specific (heterogeneity of EC).

Recently, we have shown that chronic ITSN-1s deficiency in mouse lung in the absence of inflammation and thus without GrB cleavage of ITSN-1 impedes TGFβ receptor 1/Smad-2,3/SARA (Smad anchor for receptor activation)-dependent signaling (22) to exert an EC proproliferative response. Thus, ITSN-1s deficiency may further augment the proliferative effects of the GrB-EHITSN and contribute to PLs and vascular remodeling in severe PAH.

Under physiological conditions, the concurrent expression of the cleavage products results in a high p38/Erk1/2MAPK activity ratio, which is critical for EC proliferation. It is possible that some ECs may take advantage of the growth potential, overcome death, and become hyperproliferative. The complexity of the cellular signaling and the temporal patterns of activation and regulatory feedback structures within the signaling network may further influence the p38/Erk1/2 activity ratio (40). The opposing effects of p38 activation on Erk1/2 (44) may further contribute to the high p38/Erk1/2MAPK activity ratio (Fig. 9). Increasing evidence implicates p38MAPK in the cell proliferation and vascular obliteration that characterize PAH (25, 25713).
that ITSN-1s down-regulation, the presence of the N-terminal EHITSN fragment with EC proliferative potential, and a high p38/Erk1/2 activity ratio are hallmarks of the stable EC plexiform phenotype in severe PAH.

The use of disease-free lung tissue as a control is challenging for cell signaling studies. Donor lung preservation from the time of procurement up until implantation in the recipient requires, in addition to the optimal preservation solution, storage temperature, inflation volume, and oxygen concentration, the use of pharmacological additives (i.e. prostaglandins and glucocorticoids) to enhance lung graft success (50–52). These pharmacological additives, lung transplant rejection, or perhaps associated disease may interfere with p38/Erk1/2 activity (53). Thus, the extent of kinase activation in these FD lungs should be cautiously read and used.

Another novel finding of this study is that we identified Elk-1 transcription factor as a specific downstream target of GrB-EHITSN-activated p38MAPK. We and others (20) have noted that the EH domains of ITSN activate Elk-1, a member of the Ets (E twenty-six) ternary complex transcription factors known to stimulate the expression of immediate early response genes involved in cellular proliferation and apoptosis (56). Elk-1 is a target for both the mitogenic (Erk1/2) and stress-activated (p38 and JNK) mitogen-activated protein kinases. p38MAPK is generally known as a stress kinase believed to mediate cell death (57, 58). Previous studies (59) and our work indicate, however, that p38 signaling may also be essential for cell survival and proliferation instead of death. Noteworthy is the fact that all bone morphogenetic protein type II receptor mutations underlying primary PAH demonstrate a gain of function involving up-regulation of p38MAPK-dependent proliferative pathways (55). Elk-1 positively regulates c-fos promoter activities (56, 60). The rapid induction of the c-fos gene is one of the initial events in stimulating cell proliferation by a variety of growth factors (61). Although our studies convincingly demonstrate a novel pathogenic ITSN-1s/p38-dependent signaling pathway accounting for the plexogenic EC phenotype in severe PAH, studies are still needed to show that ITSN-1s deficiency and the presence of EHITSN induce PAH in vivo.

In summary, we have demonstrated that ITSN-1s deficiency and the GrB cleavage fragment with EC proliferative potential are important players in a novel p38MAPK/Elk-1-dependent molecular mechanism underlying EC proliferation and abnormal vascular remodeling that characterizes PAH. Drugs preventing the GrB/ITSN cleavage or reducing the proliferative effect of GrB-EHITSN can be used in combination therapies for specifically targeting this normally fatal disease with multifactorial etiology.

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