Pyk2/CAKβ Tyrosine Kinase Activity-mediated Angiogenesis of Pulmonary Vascular Endothelial Cells*

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Endothelial cell spreading, migration, and morphogenesis are essential for angiogenesis, the formation of new blood vessels. In the present study, we explored roles of tyrosine kinase Pyk2 in angiogenesis of pulmonary endothelial cells. We found that tyrosine kinase Pyk2 was particularly enriched in pulmonary vascular endothelial cells and lung, a major organ site for tumor metastasis. By using adenovirus-mediated expression of various Pyk2 mutants, we demonstrated that Pyk2 tyrosine kinase activity was essential for the pulmonary vascular endothelial cell spreading, migration, morphogenesis, as well as pulmonary vein and artery angiogenesis ex vivo. We further showed that Pyk2 kinase activity was required for the expression of focal adhesion kinase, p130Crk-associated substrate, and its homologue human enhancer of filamentation 1, thus regulating formation of focal adhesions and cytoskeletal reorganization. These results indicate that Pyk2 plays a crucial role in the pulmonary endothelial cell motility such as spreading and migration necessary for angiogenesis.

Intensive studies have indicated that both tumor growth and metastasis to distant organs are dependent on angiogenesis, the formation of new blood vessels. Anti-angiogenic therapy is currently believed to be a promising approach to cancer treatment (1, 2). Angiogenesis is promoted by angiogenic factors such as basic fibroblast growth factor and vascular endothelial growth factor. Because tumor cells produce a wide array of angiogenic factors, it has been suggested that the best strategy for blocking angiogenesis is to repress the ability of endothelial cells to participate in the angiogenic process such as endothelial cell spreading and migration, rather than to prevent tumor cells from producing one particular angiogenic factor (3). However, an effective approach specifically targeting endothelial cell spreading and migration in a tissue-specific manner is still not available.

Proline-rich tyrosine kinase 2 (Pyk2)† (also called CAKβ for cell adhesion kinase β, RFTK for related adhesion focal tyrosine kinase, or CADTK for calcium-dependent tyrosine kinase) is a non-receptor tyrosine kinase structurally related to focal adhesion kinase (FAK) (4–7). Pyk2 interacts with Src family kinases and focal adhesion proteins such as p130Crk-associated substrate (p130Cas) and its homologue p105 human enhancer of filamentation 1 (HEF1) (8). HEF1 and p130Cas are multiple-domain docking proteins that can be phosphorylated by Pyk2 (9) and play important roles in integrin-mediated cytoskeletal organization and cell migration (10–14). Besides a role in the activation of mitogen-activated protein kinase, recent studies show that Pyk2 is critical for cell spreading and migration of osteoclasts (15) and monocytes (16), although the mechanisms involved are not clear. There is so far no study regarding the role of Pyk2 in angiogenesis.

In the present study, we found that Pyk2 was particularly enriched in pulmonary vascular endothelial cells and lung, a major organ site for tumor metastasis. By using adenovirus-mediated expression of various Pyk2 mutants, we demonstrated that Pyk2 tyrosine kinase activity was essential for the pulmonary vascular endothelial cell spreading, migration, morphogenesis, as well as pulmonary vein and artery angiogenesis ex vivo. Furthermore, our data indicate that Pyk2 exerts its physiological role in angiogenesis of the pulmonary endothelial cell by specifically regulating the expression levels of FAK, p130Cas, and HEF1. These findings suggest that Pyk2 plays a crucial role in the pulmonary endothelial cell motility such as spreading and migration necessary for angiogenesis.

**Experimental Procedures**

Antibodies—Monoclonal antibodies against Pyk2, FAK, phosphotyrosine (PY20), p130Cas and its homologue HEF1, paxillin, integrin β1, integrin β4, and caspase-3 were obtained from BD Transduction Laboratories (Lexington, KY). Polyclonal antibodies against integrins α5, αv, tyrosine phosphatase SHP-2, caspase-3, intracellular adhesion molecule-1, vascular cell adhesion molecule-1, and monoclonal antibodies against FAK (H-1) were obtained from Santa Cruz (CA). Monoclonal antibodies to actin and vinculin were purchased from Sigma. Monoclonal antibody to α-tubulin (AB-1) was obtained from Oncogene (Boston, MA). Cy3- and Cy5-conjugated goat antimouse and anti-rabbit antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Alkaline phosphatase-conjugated secondary antibodies and reagents for chemiluminescence detection were purchased from New England Biolabs.

Cells—Rat pulmonary vein endothelial cells were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS) and grown in a CO2 incubator at 37 °C as described previously (17). Pulmonary vein smooth muscle cells were prepared from the pulmonary vein of 12-week-old smooth muscle cell; FBS, fetal bovine serum; EGF, epithelial growth factor; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; HUVEC, human umbilical vein endothelial cells; BSA, bovine serum albumin; Ad, adenovirus.
Subcultured smooth muscle cells from passages 3 to 15 were used, which showed 99% positive immunostaining of smooth muscle α-actin and nonspecific immunostaining of melanomyoblastoma cells, NIH-3T3 mouse fibroblasts, and Chinese hamster ovary cells were purchased from American Type Culture Collection (Manassas, VA) and cultured according to the supplier’s directions. Human umbilical vein endothelial cells and human lung microvascular endothelial cells were obtained from Clonetics (Walkersville, MD).

Pyk2 in Vitro Kinase Assay—Pyk2 kinase activity was determined using exogenous substrate poly(Glu-Tyr) (4:1) (Sigma) as described previously (25). Equal amounts of lysates from recombinant adenovirus-infected cells were incubated with monochromogenic antibody anti-Pyk2 antibody. The immune complexes were collected and washed three times with the Nonidet P-40 lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2.5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10% glycerol, 100 mM NaF, 1 mM Na3VO4, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml each of leupeptin and aprotinin). The homogenates were centrifuged at 15,000 × g at 4 °C for 20 min to obtain clarified lysates. For preparing cell lysates, cells were washed twice with ice-cold phosphate-buffered saline (PBS), containing 1 mM Na3VO4, and then lysed in the Nonidet P-40 (40 μg buffer (25 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 10 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each of leupeptin and aprotinin). The extract was clarified by centrifugation at 15,000 × g at 4 °C for 10 min as described previously (17). Tissue extracts or whole cell lysates were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were probed with various primary antibodies as indicated and detected using the ECL system with alkaline phosphatase-conjugated secondary antibodies according to the manufacturer’s protocol.

Tissue Culture—Matrigel (Becton Dickinson Labware) was dried on glass cover slips and incubated at 37 °C with 5% CO2. Pulmonary vein and artery were excised from 12-week-old male Balb/c by the aortic arch. The pulmonary vein and artery were sectioned into 1–2-mm-long cross-sections and rinsed 6 times with serum-free EBM medium (Clonetics Corp.). One vein or artery ring was placed with the lumen oriented horizontally on the center of each Matrigel-coated well, covered with an additional 250 μl of Matrigel, and allowed to gel for 30–45 min at 37 °C with 5% CO2. Pulmonary vein and artery were excised from 12-week-old male Balb/c mice. The number of spread cells were counted in at least five fields.

Cell Migration Assay—Cell migration assay was determined employing a Boyden chamber assay. Polycarbonate filters (8 μm pore size, Costar) were coated with 10 μg/ml fibronectin overnight at 4 °C. The coated filters were air-dried and placed over the lower chambers that had been filled with 210 μl of serum-free medium or medium supplemented with 10% FBS or 25 ng/ml epithelial growth factor (EGF). The gasket and upper part of the chamber were then assembled. Cells infected with replication-defective recombinant adenovirus were collected by trypsinization, washed, and resuspended in serum-free medium. To obtain clear data, the resuspended cells should be passed through a 70-μm cell strainer (Falcon) to remove aggregated cells. Cells at 105 cells in 0.2 ml were added to the upper chamber. After incubation for 6 h at 37 °C, cells on the under side of the filters were mechanically removed, and the filters were fixed in methanol. Migrated cells on the under side of filters were stained with Giemsa stain (Sigma) and enumerated under a microscope at a total magnification of ×200. Five random microscopic fields were counted; all experiments were performed in duplicate.

Pulmonary Vein and Artery Rings Angiogenesis Assay—Tissue culture plates (12-well) were covered with 250 μl of Matrigel (Becton Dickinson Labware) and allowed to gel for 30–45 min at 37 °C with 5% CO2. Pulmonary vein and artery were excised from 12-week-old male Balb/c mice. The rings were cultured for 24 h in 2 ml of serum-free EBM supplemented with penicillin (100 μg/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml). After the 24-h incubation, the medium was removed, and the rings were infected with replication-defective recombinant adenovirus (1 × 109 plaque-forming units) on days 1 and 3. The cultures were kept incubated in EBM containing 2% FBS and 10 μg/ml gentamicin with medium changes every 2–3 days. Endothelial sprouts were observed and photographed on day 5 at a magnification of ×40.

Immunofluorescence Microscopy—PVECs grown overnight on glass coverslips in 6-well plates were infected with replication-defective recombinant adenovirus, washed twice with PBS, and fixed in 3.7% formaldehyde solution in PBS for 10 min at room temperature. The fixed cells were extracted in ice-cold acetone at −20 °C for 5 min, washed, and preincubated in PBS containing 1% BSA for 30 min at room temperature. For F-actin staining, coverslips were incubated for 20 min at room temperature with BODIPY® FL phallacidin (Molecular Probes, Eugene, OR) at 1.2 units per coverslip in PBS containing 1% BSA and 0.1% Triton X-100 for 30 min at room temperature. Non-BSA binding sites were then blocked by incubation with 10 μg/ml bovine serum albumin for 1 h at 37 °C. PVECs infected with replication-defective recombinant adenovirus were trypsinized, collected by centrifugation, washed once with serum-free medium, and resuspended in serum-free medium. The resuspended cells were passed through a 70-μm cell strainer (Falcon) to remove aggregated cells. Cells were then replated at a density of 2 × 104 cells/well on fibronectin-coated 12-well plates in serum-free medium for indicated times at 37 °C in a CO2 incubator and viewed under a phase-contrast microscope, and random fields were photographed. Spread cells were defined as cells having at least three processes, not round, not containing 1% BSA, and not phase-bright, whereas nonspread cells were rounded and phase-bright. The numbers of spread cells were counted in at least five fields.

Cell Spreading on Fibronectin—PVECs infected with replication-defective recombinant adenovirus were collected by trypsinization, washed, and resuspended at a density of 1 × 105 cells/well as described above. Cells were incubated in serum-free medium for indicated times at 37 °C in a CO2 incubator and viewed under a phase-contrast microscope, and random fields were photographed. Spread cells were defined as cells having at least three processes, not round, not containing 1% BSA, and not phase-bright, whereas nonspread cells were rounded and phase-bright. The numbers of spread cells were counted in at least five fields.

Pyk2-mediated Angiogenesis of Pulmonary Endothelial Cells

Sprague-Dawley rats (Charles River Laboratories) by the explant method and cultured in Dulbecco’s modified Eagle’s medium containing 10% FBS, penicillin, and streptomycin as described previously (18). Subcultured smooth muscle cells from passages 3 to 15 were used, which showed 99% positive immunostaining of smooth muscle α-actin and nonspecific immunostaining of melanomyoblastoma cells, NIH-3T3 mouse fibroblasts, and Chinese hamster ovary cells were purchased from American Type Culture Collection (Manassas, VA) and cultured according to the supplier’s directions. Human umbilical vein endothelial cells and human lung microvascular endothelial cells were obtained from Clonetics (Walkersville, MD).

In Vitro Angiogenesis Assay—The in vitro angiogenesis was determined using the measurement of capillary-like structures of endothelial cells in Matrigel (Becton Dickinson Labware). Endothelial cells infected with replication-defective recombinant adenovirus were collected by trypsinization, washed, and resuspended in serum-free medium. To obtain clear data, the resuspended cells should be passed through a 70-μm cell strainer (Falcon) to remove aggregated cells. Cells at 105 cells in 0.2 ml were added to the upper chamber. After incubation for 6 h at 37 °C, cells on the under side of the filters were mechanically removed, and the filters were fixed in methanol. Migrated cells on the under side of filters were stained with Giemsa stain (Sigma) and enumerated under a microscope at a total magnification of ×200. Five random microscopic fields were counted; all experiments were performed in duplicate.

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and brain (Fig. 1, 3rd panel). Focal adhesion proteins p130Cas and HEF1 are most abundant in lung and brain (Fig. 1A). On the other hand, vinculin were detected in all tissues. Thus, Pyk2 and FAK were barely detectable in NIH-3T3 fibroblasts, B16 mouse melanoma cells, and Chinese hamster ovary cells (CHO). In agreement with previous reports (6, 24), Pyk2 was highly expressed in pulmonary vascular cells. Total cell lysates from pulmonary vein smooth muscle cells (PVSMC), human lung microvascular endothelial cells (HMVEC-L), human umbilical vein endothelial cells (HUVEC), NIH-3T3 fibroblasts, B16 mouse melanoma cells, and Chinese hamster ovary cells (CHO) were subjected to immunoblotting with antibodies to Pyk2 and FAK. Abundant Pyk2 protein level in rat lung and brain. Adult rat tissue homogenates were subjected to immunoblotting with indicated antibodies. Results shown are representative immunoblots of three independent experiments.

RESULTS

High Protein Level of Pyk2 in Pulmonary Vascular Endothelial Cells and Lung—Unlike the nearly widespread distribution of FAK, Pyk2 expression is highly cell type- and tissue-specific (6, 24). As shown in Fig. 1A, the highest amount of Pyk2 was detected in rat pulmonary vein endothelial cells (PVEC). A high level of Pyk2 protein was also detected in pulmonary vein smooth muscle cells (PVSMC). Moreover, Pyk2 was detected in human lung microvascular endothelial cells (HMVEC-L) but not in human umbilical vein endothelial cells (HUVEC). In agreement with previous reports (6, 24), Pyk2 was barely detectable in NIH-3T3 fibroblasts, B16 mouse melanoma cells, and Chinese hamster ovary cells (CHO-K1). In contrast, a similar amount of FAK was detected in all cell types (Fig. 1A). Thus, Pyk2 was highly expressed in pulmonary vascular endothelial cells such as PVEC.

To gain possible insight into the pathophysiologic significance of Pyk2, we determined the protein level of Pyk2 in adult rat tissues. As shown in Fig. 1B (top), the Pyk2 protein level was particularly abundant in lung and brain. Pyk2 protein level was very low in aorta, adrenal gland, and kidney and was below the detectable threshold in heart and liver. In contrast, FAK was detected in all tissues except liver (Fig. 1B, 2nd panel). Immunoblot analysis using a monoclonal antibody, which recognizes p130Cas and its homologue HEF1 (9), revealed that p130Cas and HEF1 were highly expressed in lung and brain (Fig. 1B, 3rd panel). On the other hand, the cell survival kinase Akt and a similar amount of the cytoskeletal protein vinculin were detected in all tissues. Thus, Pyk2 and focal adhesion proteins p130Cas and HEF1 are most abundant in the lung and brain.

Effects of Overexpression of Pyk2 Mutants on PVEC Morphology—Because data obtained from a “gain of function” study by expressing a signaling molecule in cells lacking the protein may not really reflect the molecular physiological function, we performed a “loss of function” study by expressing Pyk2 dominant negative mutants in PVEC expressing endogenous Pyk2. To gain a sufficient expression level of Pyk2 mutants, we generated replication-defective recombinant adenovirus expressing catalytically inactive Pyk2 mutant K457A (AdPyk2-K457A), Pyk2-Y402F (AdPyk2-Y402F), Pyk2 wild type (AdPyk2), as well as control cosmid pAXCAwt only (Ad vector) according to the COS-TPC method (21). The mutants were then characterized and confirmed in NIH-3T3 cells that do not express endogenous Pyk2. We found that mutation of the ATP-binding site lysine 457 to alanine completely abolished Pyk2 kinase activity as assessed by phosphorylation of the substrate poly(Glu-Tyr) (4:1), confirming that Pyk2-K457A is a catalytically inactive mutant. Mutation of the principal autophosphorylation site tyrosine 402 to phenylalanine only slightly inhibited (22%) Pyk2 kinase activity toward poly(Glu-Tyr) (4:1). These results are consistent with a recent report (22) using 293 cells. PVECs at subconfluence were then infected with AdPyk2-Y402F, AdPyk2-K457A, AdPyk2, or Ad vector for 16 h, and cell morphology was observed. As shown in Fig. 2A, expression of Pyk2 wild type did not affect PVEC morphology but slightly increased the rate at which cells reach confluence as compared with cells infected with Ad vector control. Overexpression of Pyk2-Y402F slowed the rate at which cells reach confluence but did not affect cell morphology, probably due to inhibition of cell growth (5, 25). Interestingly, when the catalytically inactive mutant Pyk2-K457A was overexpressed, PVECs were aggregated, became small in size, and showed a rounded morphology (Fig. 2A). We also found that the aggregated PVECs adhered to a culture dish at a low strength because they can be relatively easily detached by aspiration compared with control cells. The effect was dependent on the infection dose or expression level of Pyk2-K457A and can be observed as early as 4 h post-infection and reached the maximal level between 12 and 24 h after infection (data not shown). As expected, overexpression of Pyk2-K457A did not affect cell morphology and cell confluence in NIH-3T3 and HUVEC cells that lack endogenous Pyk2 (Fig. 2B). Fig. 2C shows expression levels of the Pyk2 mutants in PVEC, NIH-3T3, and HUVEC. These data suggest that the physiological function of Pyk2 kinase activity is to regulate PVEC motility or cell-cell adhesion.

Pyk2 Kinase Activity Is Required for PVEC Spreading and Migration—We then investigated the effect of Pyk2-K457A on PVEC motility. We first examined PVEC viability. We found that over 99% PVECs treated with replication-defective recombinant adenovirus expressing Pyk2-K457A and other Pyk2 mutants were stained negative with trypan blue, indicating that they were alive. We also found that none of the Pyk2 mutants affected the activity of caspase-3, a critical enzyme in the process of apoptosis. These data indicate that none of the Pyk2 mutants affect the viability of PVEC. PVEC adhesion to fibronectin and spreading were then determined. Overexpression of the catalytically inactive mutant K457A markedly suppressed (78%) PVEC spreading, whereas it had only a minor effect on PVEC adhesion at 5 and 10 min (Fig. 3, A and B). Neither the Pyk2 wild type nor Y402F mutant affected PVEC adhesion and spreading. Because cell adhesion-mediated spreading is a basis for cell migration, we determined the effect of Pyk2-K457A on PVEC migration. Cell migration was determined employing a Boyden chamber assay. As shown in Fig. 3C, PVEC migration evoked by 10% FBS and EGF (25 ng/ml) were almost completely inhibited by overexpression of the catalytically inactive mutant Pyk2-K457A. We also found that overexpression of Pyk2-K457A markedly inhibited cell migration of PVSMCs, which are the important component of vascular wall and express endogenous Pyk2. As expected, Pyk2-
K457A did not affect the migration of NIH-3T3 lacking endogenous Pyk2 (data not shown). These results indicate that Pyk2 kinase activity is essential for PVEC spreading and migration.

Pyk2 Kinase Activity Is Critical for the Formation of Capillary-like Structure by PVEC—Endothelial cell adhesion and migration are essential for multiple steps of the angiogenic process. Later stages of angiogenesis require morphological alterations of endothelial cells, which result in lumen formation (28). We performed an in vitro model of angiogenesis in which PVECs are induced to form a network of capillary-like tubes by angiogenic factors, and we determined the effect of Pyk2 kinase activity on this process. As shown in Fig. 4A, treatment with an angiogenic factor PMA (100 nM) for 4 h clearly induced a network of capillary-like structure in PVEC infected with Ad vector. However, the PMA-induced capillary-like tube formation was virtually abolished by overexpression of Pyk2-K457A in PVEC (Fig. 4A). Similar capillary-like structure formation stimulated by 10% FBS for 4 h was also virtually abolished by the Pyk2 catalytically inactive mutant (data not shown).

Pyk2 Kinase Activity Is Critical for Pulmonary Vein and Artery Angiogenesis ex Vivo—We next determined the effects of Pyk2 kinase activity on angiogenesis using an ex vivo model with intact vascular explants, which may be closer to the in vivo condition than that with isolated endothelial cells. We investigated ex vivo angiogenesis using rings of rat pulmonary vein or artery as a source of vasoformative endothelial cells. Pulmonary vein rings are soft and readily collapse; thus it is hard to place them with the lumen oriented horizontally in Matrigel. As shown in Fig. 4B, dense vessel sprouting was observed from pulmonary vein rings infected with the Ad vector control. However, the vessel sprouting from the vein ring was markedly suppressed by infection with AdPyk2-K457A. A similar effect was observed when pulmonary artery angiogenesis assay was employed. Pulmonary arterial rings are rigid and can be placed with the lumen oriented horizontally in Matrigel. As shown in Fig. 4B (bottom), the vessel sprouting from the vein ring was markedly suppressed by infection with AdPyk2-K457A. A similar effect was observed when pulmonary artery angiogenesis assay was employed. Pulmonary arterial rings are rigid and can be placed with the lumen oriented horizontally in Matrigel. As shown in Fig. 4B, the vessel sprouting from the vein ring was markedly suppressed by infection with AdPyk2-K457A.

Pyk2 Kinase Activity Specifically Regulates the Expression of FAK, p130 Cas, and HEF1—Because overexpression of Pyk2-K457A suppressed PVEC spreading and migration, it suggests that Pyk2 kinase activity may be required for the expression of focal adhesion proteins or the formation of focal adhesions. Subconfluent PVECs were infected with AdPyk2-K457A or other mutants overnight, and equal amounts of cell lysates
were subjected to immunoblotting with indicated antibodies (Fig. 5). We found that overexpression of Pyk2-K457A markedly suppressed (70%) FAK protein level (Fig. 5A, left top), and this effect was not due to the degradation of FAK because no degraded products of FAK were detected by FAK antibodies against the FAK kinase domain (Fig. 5B) and C terminus (not shown). Furthermore, we found that the p130\(^{Cas}\) level was almost completely (90%) suppressed in PVEC overexpressing Pyk2-K457A. This effect was also not due to the degradation of p130\(^{Cas}\) because no degraded products were detected by a p130\(^{Cas}\) antibody (Fig. 5B). It has been reported that the antibody to p130\(^{Cas}\) detects both p130\(^{Cas}\) and its homologue p105HEF1 (9). Whereas HEF1 was detected as a 105-kDa protein band below p130\(^{Cas}\) in PVEC, overexpression of Pyk2-K457A abolished HEF1 expression (Fig. 5). In contrast, overexpression of Pyk2-K457A did not affect significantly the levels of vinculin, a cytoskeletal protein that localizes at focal adhesion patches (Fig. 6B). On the other hand, Pyk2 wild type, Pyk2-Y402F, as well as vector alone did not affect the expression of integrins, focal adhesion proteins, and SHP-2 as tested above (Fig. 5A). As expected, neither of the Pyk2 mutants affected the expression of integrins nor focal adhesion proteins tested above in NIH-3T3 fibroblasts lacking endogenous Pyk2 (Fig. 5A, right). This may account for the absence of effects of Pyk2-K457A on NIH-3T3 cell spreading and migration. Taken together, these findings indicate that Pyk2 kinase activity is critical and specifically regulates the cellular expression of focal adhesion proteins FAK, p130\(^{Cas}\), and HEF1 in PVEC.

Pyk2 Kinase Activity Is Critical for Formation of Focal Adhesions and Actin Stress Fibers—Cell adhesion to extracellular matrix (ECM) is a highly dynamic process involving structures heterogeneous with respect to size, composition, and orientation to actin filaments. The largest and tightest ones are usually referred to as focal adhesions, which link the actin cytoskeleton to ECM by integrin receptor complexes (27). Because FAK, p130\(^{Cas}\), and HEF1 are important components of focal adhesion complexes and they are essential for the formation of focal adhesion contacts (13, 28), our data suggest that overexpression of Pyk2-K457A may affect the formation of focal adhesions in PVEC. Cells grown overnight on glass coverslips were infected with replication-defective recombinant adenovirus expressing Pyk2 wild type (WT), Pyk2 mutants (Y402F and K457A) or vector alone for 16 h, and cell lysates were subject to immunoblotting with the indicated antibodies. B, overexpression of Pyk2-K457A did not induce degradation of FAK and p130\(^{Cas}\) in PVEC. Results shown are representative immunoblots of three independent experiments.

Because focal adhesions link the actin cytoskeleton to ECM, we determined effects of Pyk2-K457A and other mutants on formation of actin stress fibers using BODIPY® FL phalloidin in PVEC. Clear actin stress fibers that cross the cell body and...
contacts. Actin stress fibers were stained by BODIPY (1:120 dilution), a cytoskeletal protein that localizes at focal adhesion.

Viewed by staining the fixed cells with monoclonal antibody to vinculin.

K457A or vector alone, washed twice, and fixed. Focal contacts were binant adenovirus expressing Pyk2 catalytically inactive mutant.

night on glass coverslips were infected with replication-defective recom-

gence of cellular adhesions and actin stress fibers in PVEC.

A, PVEC grown over-

immunoblots of actin and vinculin in PVEC overexpressing Pyk2-K457A or vector alone. Results shown are repre-

sentatives of three independent experiments.

condense at the cell periphery were observed in control PVEC (Fig. 6A, left lower panel) or PVEC overexpressing wild type or Pyk2-Y402F (data not shown). In contrast, the typical pattern of actin stress fibers was not detected in PVEC overexpressing Pyk2-K457A. Similar to the shift of vinculin localization as shown above, actin, in most cases, was mainly detected at cell-cell adhesion regions (Fig. 6A, right lower panel). Immuno-

blotting analysis revealed that overexpression of Pyk2-K457A did not affect the expression of actin in PVEC (Fig. 6B). Thus, Pyk2 kinase activity is critical for adhesion-dependent cytoskeletal reorganization in PVEC.

DISCUSSION

The role of tyrosine kinase Pyk2 in angiogenesis has not been reported to date. In the present study, we have investigated the physiologic function of Pyk2 in pulmonary endothelial cells using adenovirus-mediated expression of various Pyk2 mutants. We found that Pyk2 was enriched in PVEC, and Pyk2 kinase activity was essential for the endothelial cell spreading, migration, morphogenesis, as well as pulmonary vein and ar-

tery angiogenesis ex vivo. Furthermore, we identified that Pyk2 kinase activity was critical for the cellular expression of FAK, p130Cas, and HEF1, the formation of focal adhesions, and cytoskeletal reorganization in PVEC. Pyk2 could be an important regulator of pulmonary angiogenesis.

Pyk2/CAKa is a non-receptor tyrosine kinase structurally related to FAK (4–7). Unlike the nearly widespread distribution of FAK, Pyk2 expression is highly cell type- and tissue-
specific (6, 24). We found that Pyk2 protein was particularly abundant in lung and pulmonary vascular endothelial cells but was barely detectable in HUVEC, NIH-3T3 fibroblasts, B16 melanoma, and CHO cells. Ridyard and Sanders (30) recently reported that Pyk2 was hard to detect in early embryo stages, whereas FAK was highly expressed before embryonic gastrula-

tion. This result suggests that the expression of Pyk2 and FAK is independently regulated. Because Pyk2 is selectively expressed in pulmonary endothelial cells and lung, a major organ for tumor metastasis, the present findings suggest that a Pyk2 inhibitor might be a good candidate to target tumor-induced pulmonary angiogenesis in order to arrest tumor growth and metastasis.

Although Pyk2 and FAK are highly homologous (45%) in primary structure and share several functional domains, recent studies (29, 31, 32) have shown that Pyk2 and FAK are differentially regulated and appear to have distinct physiologic functions as well. Pyk2 is mainly distributed diffusely throughout the cytoplasm and concentrated in the perinuclear region, and is readily activated by a wide variety of agonists that increase intracellular calcium concentration or activate protein kinase C in non-hematopoietic cells (5, 17, 29, 31, 32), whereas FAK localizes predominantly to focal adhesion contacts in ad-

herent cells and is rapidly tyrosine-phosphorylated and activated upon integrin-mediated cell adhesion (27). Down-regula-

tion of Pyk2 by expressing a Pyk2 antisense or the C-terminal noncatalytic domain of Pyk2 suppresses cell spreading and migration of osteoclasts (15) and monocytes (16), respectively. These results indicate that Pyk2 plays an important role in cell motility, although the mechanisms are not clear. On the other hand, overexpression of Pyk2 does not rescue the cell migratory defects caused by FAK deletion in FAK-deficient fibroblasts (29). This indicates that the physiologic role of Pyk2 is distinct from FAK, and it also suggests that Pyk2 may regulate cell motility through FAK. Here we found that overexpression of a catalytically inactive Pyk2 mutant K457A but not other mutants suppressed the angiogenic processes (cell spreading, migration, and morphogenesis) of PVEC. Furthermore, overex-

pression of the Pyk2 mutant K457A specifically down-regulated FAK, p130Cas, and HEF1 and disrupted the formation of focal adhesion and cytoskeletal reorganization in PVEC. These findings suggest that Pyk2 exerts its physiologic role in the angiogenesis of PVEC by specifically regulating, via its kinase activity, the expression of critical components of focal contacts such as FAK, p130Cas, and HEF1. In addition, it is of interest to know the mechanisms that regulate the expression of these critical focal adhesion molecules in cells like HUVEC lacking endogenous Pyk2.

Several lines of evidence suggest that Cas proteins such as p130Cas and HEF1 may be the key regulators of actin cytoskel-

etal reorganization (13) and are required for FAK-mediated cell migration (10, 12). It has been reported that p130Cas plays an important role in actin organization in osteoclasts (33). Fur-

thermore, p130Cas-deficient embryo dies in utero showing marked systemic congestion and growth retardation, and fibro-

blasts from the embryo display severely impaired actin stress fiber formation and migration (11). We found that p130Cas and HEF1 were particularly abundant in adult rat lung and brain compared with other tissues. They were also highly expressed in PVEC (not shown). Overexpression of the catalytically inactive Pyk2 mutant K457A virtually completely down-regulated p130Cas and HEF1, which was correlated with the severe dis-

ruption of actin stress fibers in PVEC. These findings indicate that Pyk2 kinase activity is critical for expression of the Cas proteins and cytoskeletal reorganization. Our data also support the concept that Cas proteins are involved in actin cytoskeletal reorganization.

Autophosphorylation of Pyk2 on tyrosine 402 and subsequent interaction with Src family kinase have been shown to play an important role in the activation of mitogen-activated protein kinase, thus regulating cell proliferation (22, 25). We found that overexpression of Pyk2-Y402F in PVEC delayed cells attaining confluence compared with control cells. This
may be due to the inhibition of cell growth. It has been shown that mutation of the Pyk2 principal autophosphorylation site tyrosine 402 to phenylalanine markedly suppressed Pyk2 autophosphorylation but had only a minor effect on Pyk2 kinase activity, whereas mutation of Pyk2 ATP-binding site lysine 457 to alanine abolished Pyk2 kinase activity and autophosphorylation in 293 cells (22). Similar results were obtained in NIH-3T3 cells lacking endogenous Pyk2 in the present study. These data indicate that Pyk2 kinase activity is essential for the autophosphorylation of tyrosine 402. It seems that the phenotype end points observed in PVEC expressing Pyk2-K457A may result from both the inhibition of phosphorylation of Pyk2 substrates like Cas proteins and the inhibition of tyrosine 402-mediated interaction with critical signal molecules like Src family kinases.

Finally, we want to point out an interesting observation. We found that the typical vinculin patches at focal adhesion sites and the formation of actin stress fibers could not be detected in PVEC-overexpressing Pyk2-K457A. Instead, we found that vinculin and F-actin were mainly detected at cell-cell adhesion regions. Focal adhesions are major sites that link the actin cytoskeleton to ECM by integrins. Specific classes of integrins also mediate important cell-cell adhesive interactions (27). Actin-binding proteins that co-localize with integrins in focal adhesions include α-actinin, talin, vinculin, and tensin. Thus, actin filaments can link to cytoplasmic domains of integrin through these actin-binding proteins. Inhibition of Pyk2 kinase activity suppressed the expression of FAK and Cas proteins and disrupted the formation of focal adhesion contacts and actin reorganization at the focal contacts. This may render the binding of vinculin and actin to specific integrins at cell-cell adhesion region, thus changing cellular force direction. This may be one of the mechanisms that cause PVEC to aggregate when Pyk2 kinase activity is inhibited. In addition, we found that overexpression of Pyk2-K457A did not affect the expression of integrins (Fig. 5), and cell adhesion molecules such as vascular cell adhesion molecule-1 and intracellular adhesion molecule-1 (data not shown), which are ligands for specific integrins, were not upregulated.

In summary, Pyk2 is selectively expressed in lung and pulmonary vascular endothelial cells, and its kinase activity is essential for the endothelial cell angiogenesis process. Our findings suggest that Pyk2 is a critical regulator of pulmonary angiogenesis.

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