Receptor for Activated C-Kinase 1, a Novel Interaction Partner of Type II Bone Morphogenetic Protein Receptor, Regulates Smooth Muscle Cell Proliferation in Pulmonary Arterial Hypertension

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Background—Pulmonary arterial hypertension (PAH) is characterized by selective elevation of pulmonary arterial pressure. The pathological hallmark of PAH is the narrowing of pulmonary arterioles secondary to endothelial cell dysfunction and smooth muscle cell proliferation. Heterozygous mutations in BMPR2, encoding the type II bone morphogenetic protein receptor (BMPRII), were identified in PAH, suggesting that alterations to BMPRII function are involved in disease onset and/or progression.

Methods and Results—We identified the receptor for activated C-kinase (RACK1) as a novel interaction partner of BMPRII by yeast 2-hybrid analyses using the kinase domain of BMPRII as a bait. Glutathione-S-transferase pull-down and coimmunoprecipitation confirmed the interaction of RACK1 with BMPRII in vitro and in vivo. RACK1–BMPRII interaction was reduced when kinase domain mutations occurring in patients with PAH were introduced to BMPRII. Immunohistochemistry of lung sections from PAH and control patients and immunofluorescence analysis of primary pulmonary arterial smooth muscle cells demonstrated colocalization of BMPRII and RACK1 in vivo. Quantitative reverse-transcription polymerase chain reaction and Western blot analysis showed significant downregulation of RACK1 expression in the rat model of monocrotaline-induced PAH but not in pulmonary arterial smooth muscle cells from PAH patients. Abrogation of RACK1 expression in pulmonary arterial smooth muscle cells led to decreased Smad1 phosphorylation and increased proliferation, whereas overexpression of RACK1 led to increased Smad1 phosphorylation and decreased proliferation.

Conclusions—RACK1, a novel interaction partner of BMPRII, constitutes a new negative regulator of pulmonary arterial smooth muscle cell proliferation, suggesting a potential role for RACK1 in the pathogenesis of PAH. (Circulation. 2007;115:2957-2968.)

Key Words: cardiovascular diseases ■ hypertension, pulmonary ■ remodeling

Pulmonary arterial hypertension (PAH) is a progressive and ultimately fatal disease defined by selective elevation of the mean pulmonary arterial pressure by at least 25 mm Hg at rest or >30 mm Hg during exercise.1,2 The underlying cause of this sustained elevation is an increased pulmonary vascular resistance, resulting in progressive right heart hypertrophy, reduced right heart function, and heart failure caused by increased right ventricular afterload.1-3-5 A key event in the development of PAH is pulmonary vascular remodeling, a complex process involving all layers and cells of the vessel wall.6,7 The pathological hallmark of vascular remodeling in PAH is the progressive narrowing and obstruction of small pulmonary arteries as a result of changes in the structure and function of cells located within the vessel wall (including endothelial and smooth muscle cells [SMCs], as well as adventitial fibroblasts).5,9 Structural changes that are observed routinely in PAH include vascular cell hypertrophy, hyperplasia, and an increased deposition of extracellular matrix proteins (including collagen and elastin). Although the pathological changes typical in PAH...
have been well defined, the origin of this disease remains unclear.\textsuperscript{10} In 2000, positional cloning revealed that patients affected by familial PAH exhibited germ-line mutations within the BMPR2 locus, which encodes the type II bone morphogenetic protein receptor (BMPRII).\textsuperscript{11–14} A ubiquitously expressed member of the transforming growth factor (TGF)-β receptor superfamily. To date, direct sequence analysis has identified multiple heterogeneous germ-line mutations in BMPR2 exons in \( \approx 50\% \) of familial PAH and 10% to 25% of idiopathic PAH (IPAH) patients.\textsuperscript{15,16} Most of these mutations represent missense, nonsense, or frame-shift mutations in BMPRII and are predicted to lead to a loss of function of BMPRII protein.\textsuperscript{16,17}

The BMP ligands exhibit pleiotropic effects in different cell types, including the regulation of cell proliferation, apoptosis, and differentiation, as well as tissue patterning and organogenesis in the developing embryo.\textsuperscript{18,19} BMP signaling is induced on ligand binding to the high-affinity type I BMP receptors BMPRIA (ALK3) and BMPRIB (ALK6). Type I receptors then form a heterotetrameric complex of type I and type II receptors, which phosphorylates the intracellular signaling proteins Smad1 and Smad5. Smad1 and Smad5 form complexes with Smad4, translocate to the nucleus, and regulate the transcription of BMP-responsive genes.\textsuperscript{20,21}

BMP-dependent signaling has been demonstrated to modify the proliferative response of SMCs because BMP2, BMP4, and BMP7 have been reported to inhibit vascular SMC proliferation.\textsuperscript{22–24} In families with BMPR2 mutations, this mutation causes PAH, but the exact molecular mechanism of this genotype-to-phenotype axis remains to be elucidated. It is currently hypothesized that mutations in the gene encoding BMPRII generate dysfunctional receptors that may induce proliferation of pulmonary artery SMC (paSMCs), promoting an increase in pulmonary vascular resistance and ultimately pulmonary hypertension. Although these genetic studies have assigned a causative role for BMP receptors in the development of PAH, our understanding of the functional contributions and expression of this system in the lung in general, and PAH in particular, is still evolving.

**Methods**

**Plasmid Construction**

Full-length mouse BMPRII cDNA was amplified by polymerase chain reaction (PCR) using primers containing built-in Apal restriction sites and ligated into pCMV-HA (Invitrogen, Carlsbad, Calif). A yeast 2-hybrid construct encoding the BMPRII kinase domain (residues 209 to 530) was created using primers containing built-in EcoRI and BamHI restriction sites and inserted into pGBK7T (BD Biosciences, San Jose, Calif). Full-length receptor of activated C chain (RACK)-1 was amplified by PCR and ligated into pCMV5A-Myc (Invitrogen). All primers sequences used for cloning are given in Table I in the online Data Supplement.

**Yeast 2-Hybrid Screen**

To identify novel BMPRII-interacting proteins, a yeast 2-hybrid screen was performed using the Matchmaker3 GAL4 2-hybrid system (BD Biosciences). The bait plasmid containing the BMPRII kinase domain was transformed into Saccharomyces cerevisiae strain AH109 and mated with strain Y187, which was pretransformed with an 11-day mouse embryonic cDNA library constructed in the yeast 2-hybrid vector pACT2. Diploid yeast cells were grown on high-stringency selection media (lacking the essential amino acids Leu, Trp, His, and Ade supplemented with X-Gal). Plasmids from positive yeast colonies were isolated and sequenced. All sequences obtained were compared with known transcripts in the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm (www.ncbi.nlm.nih.gov/BLAST).

**Glutathione-S-Transferase Pull-Down Assay**

A prokaryotic expression vector expressing the BMPRII kinase domain fused to glutathione-S-transferase (GST) was overexpressed in Escherichia coli BL21. Recombinant BMPRII-GST was recovered by lysis of the cells in 20 mmol/L Tris-Cl (pH 7.5), 150 mmol/L NaCl, 10 mmol/L EDTA, 5 mmol/L EGTA, 0.1% (vol/vol) β-mercaptoethanol, and 1× protease inhibitors (Complete; Roche, Mannheim, Germany). BMP-2 was incubated with glutathione-Sepharose beads (Amersham Biosciences, Uppsala, Sweden) (1.5 hours, 4°C) and, to avoid nonspecific binding, washed (3 times) with 20 mmol/L Tris-Cl (pH 7.5), 150 mmol/L NaCl, 10 mmol/L EDTA, EGTA, and 0.5% (vol/vol) Triton X-100 supplemented with 1× Complete protease inhibitors. GST-BMPRII was incubated with lysates from NIH3T3 cells overexpressing Myc-tagged RACK1 (1.5 hours, 4°C) in lysis buffer (50 mmol/L Tris-HCl [pH 7.5], 150 mmol/L NaCl, 10 mmol/L sodium pyrophosphate, 0.5% [vol/vol] NP-40, and 1× Complete protease inhibitors). After extensive washing (3 times, 1.5 mL in lysis buffer, samples were boiled for 5 minutes in 2× Laemmli sample loading buffer (60 mmol/L Tris-Cl [pH 6.8], 10% [vol/vol] glycerol, 2% SDS, 5% [vol/vol] β-mercaptoethanol, and 0.025% [vol/vol] bromophenol blue) and resolved on 12% SDS-PAGE gels.

**Site-Directed Mutagenesis**

Truncated GST-BMPRII kinase domain fusion proteins were prepared by site-directed mutagenesis of the wild-type GST-BMPRII kinase domain fusion protein using the Quick-Change site-directed mutagenesis system (Stratagene, La Jolla, Calif). Mutagenic primers carried a single nucleotide substitution identified in IPAH patients, which generated a premature stop codon at positions 1483, 1397, 1348, and 994. The primers used are listed in Table II in the online Data Supplement. All point mutations were verified by direct sequencing.

**Immunoprecipitation**

Protein G-Sepharose beads (50 μL of a 1:1 suspension in lysis buffer; Amersham Biosciences) were preincubated with anti-Myc IgG (2 μg; Cell Signaling Technology, Beverly, Mass). NIH3T3 cells overexpressing HA-tagged BMPRII and Myc-tagged RACK1 were lysed in 50 mmol/L Tris-Cl (pH 7.5), 150 mmol/L NaCl, 10 mmol/L sodium pyrophosphate, 0.5% (vol/vol) NP-40, and 1× Complete protease inhibitors. Cell extracts were then incubated with antibody-bead complexes (2 hours, 4°C). The immunoprecipitates were washed (3 times, 0.5 mL lysis buffer), resuspended in 2× Laemmli sample loading buffer, boiled (5 minutes), and resolved on 12% SDS-PAGE gels.

**Human Tissues and paSMCs**

Lung tissue samples were obtained from 12 patients with IPAH (mean age, 34.5 ± 10.5 years; 8 women, 4 men) and 9 control subjects (organ donors; mean age, 37.8 ± 14.1 years; 5 women, 4 men). None of the IPAH patients exhibited BMPR2 mutations. Samples were placed in 4% (wt/vol) paraformaldehyde within 30 minutes after explantation. The study protocol was approved by the ethics committee of the Justus-Liebig-University School of Medicine (AZ 31/93). Informed consent was obtained from each subject for the study protocol. Primary paSMCs were generated from lobar pulmonary arteries from donors or IPAH patients known to harbor a mutation in BMPR2 (n=3 for each) as described.\textsuperscript{26}

**Cell Cycle Analysis by Flow Cytometry**

To determine DNA content, cells were harvested by trypsinization 24 hours after transfection, fixed overnight at 4°C with 75% (vol/vol)
ethanol, washed, and incubated in PBS containing 10 μg/mL propidium iodide and 100 μg/mL RNase for 1 hour at 37°C. Data were acquired using a fluorescent-activated cell sorter (FACS) Canto flow cytometer and analyzed by FACS DiVa software package (BD Biosciences). A minimum of 10,000 cells were analyzed per sample. Gates based on forward and side scatter were set to eliminate cellular debris and cell clusters.23

Immunofluorescence and Immunohistochemistry
Cells were seeded in 9-well chamber slides and processed for immunofluorescence analysis as described.23 Immunohistochemical analysis of paraffin-embedded lung sections from healthy transplant donors or IPAH patients was performed as outlined.27

Western Blot Analysis
Cell extracts (20 μg) were resolved on 10% reducing SDS-PAGE gels and blotted onto nitrocellulose membranes (Bio-Rad, Hercules, Calif). Protein expression was analyzed using antibodies against the following epitopes: Myc (Cell Signaling, Danvers, Mass), HA (Sigma-Aldrich, Saint Louis, Mo), GST, pSmad1/3, or Smad1 (all from Cell Signaling). Immune complexes were visualized with horseradish peroxidase-conjugated secondary antibodies (Pierce, Rockford, Ill) using the ECL Plus system (Amersham Biosciences).23

Reverse-Transcription PCR
Total RNA was extracted from fresh-frozen lung samples using the Roti-Quick RNA extraction procedure according to the manufacturer’s instructions (Roth, Karlsruhe, Germany). RNA samples were reverse transcribed using ImProm II reverse transcriptase (RT; Promega, Mannheim, Germany). Real-time PCR was performed by the Sequence Detection System 7700 (PE Applied Biosystems, Wellesley, Mass).28,29 Signals were normalized to porphobilinogen deaminase. All primers sequences for RT-PCR are given in Table III in the online Data Supplement.

Transfection With Small Interference RNA
Four small interference RNA (siRNA) sequences directed against human RACK1 were used to attenuate RACK1 expression in paSMCs (siRNA sequences shown in Table IV in the online Data Supplement). To control for nonspecific gene inhibition of the siRNAs, a negative-control siRNA sequence was used (Ambion, Austin, Tex). Cells were transfected with siRNA (100 nmol/L) using the Basic Nucleofactor Kit (Amaxa Biosystems, Cologne, Germany). These clones were transformed into yeast pretransformed with the BMPRII kinase domain or the complete BMPRII cytosolic region fused to the Gal4-DNA–binding domain (BD). We were able to verify the interaction of BMPRII with RACK1, demonstrated by colony growth on high-stringency selective plates when DB-BMPRII kinase or DB-BMPRII total were expressed alongside AD-clone 42, AD-clone 58, AD-clone 97, and AD-RACK1 (Figure 1B).

RACK1–BMPRII Interaction In Vivo
We next sought to independently verify the BMPRII–RACK1 interaction in mammalian cells. Figure 2A presents the results of a GST pull-down assay, illustrating the specific interaction of the kinase domain of BMPRII with Myc-tagged RACK1, whereas GST alone did not interact with RACK1. Similar results were obtained in coinmunoprecipitation experiments, which further demonstrated a BMP2-independent interaction of BMPRII with RACK1 (Figure 2B). We then asked whether BMPRII mutations that have been found in IPAH patients would affect the interaction of RACK1 with BMPRII and generated 4 different BMPRII constructs containing the mutation Q495X, W466X, Q450X, or R332X (Figure 2C). Interestingly, all BMPRII variants did interact with RACK1, but their interaction was significantly weaker than wild-type BMPRII (Figure 2D), indicating that the full-length BMPRII kinase domain was required for maximal binding to RACK1. We next elucidated the effect of the shortest truncated variant of BMPRII (R332X) on paSMC proliferation by cell cycle analysis. We observed that cell proliferation was decreased when wild-type BMPRII was transfected into paSMCs, whereas it was increased if the BMPRII mutant R332X was expressed (Figure 2F). Transfection efficiency was monitored by FACS analyses of enhanced green fluorescent protein (EGFP) expression and was routinely ~50% in paSMCs (Figure 2E).

RACK1–BMPRII Colocalization in paSMCs In Vivo and In Vitro
To further investigate the expression and function of RACK1 and its interaction with BMPRII in the lung, we analyzed RACK1 protein localization in the lung. Localization of BMPRII, RACK1, and smooth muscle actin (SMA) was

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results
Identification of RACK1 as a Novel BMPRII Interaction Partner
To identify novel interaction partners of BMPRII, a yeast 2-hybrid screen was performed using the BMPRII kinase domain as a bait. This screen identified 3 overlapping clones (clones 42, 58, and 97; Figure 1A), which exhibited >97% sequence identity with RACK1, a molecule containing 7 WD domains (Figure 1A). To verify the interaction of BMPRII with these 3 clones, as well as full-length RACK1, by reverse 2-hybrid analysis, all constructs were subsequently expressed as Gal4 activation domain (AD) fusion proteins (designated AD-clone 42, AD-clone 58, AD-clone 97, and AD-RACK1). These clones were transformed into yeast pretransformed with the BMPRII kinase domain or the complete BMPRII cytosolic region fused to the Gal4-DNA–binding domain (BD). We were able to verify the interaction of BMPRII with RACK1, demonstrated by colony growth on high-stringency selective plates when DB-BMPRII kinase or DB-BMPRII total were expressed alongside AD-clone 42, AD-clone 58, AD-clone 97, or AD-RACK1 (Figure 1B).

A Monocrotaline Rat Model of PAH
Samples from the monocrotaline-induced rat model of PAH were obtained as described previously.30,31

Assessment of Cell Proliferation
Cell proliferation of paSMCs was assessed by direct cell counting and [3H]-thymidine incorporation analysis as described previously.23

Luciferase Reporter Assay
Luciferase assays were performed with the pID120 reporter construct containing a BMP-responsive element upstream of a firefly luciferase gene as previously described.26

Statistical Analysis
Values are presented as mean±SEM. The means of indicated groups were compared using 2-tailed Student t test or a 1-way ANOVA with Tukey’s highest-significant-difference post hoc test for studies with >2 groups. A level of P<0.05 was considered statistically significant.
determined by immunostaining of donor and IPAH lung tissues. As depicted in Figure 3A, BMPRII staining was demonstrated within endothelial cells and paSMCs. Similarly, RACK1 expression was localized predominantly to paSMCs and, to a lesser extent, endothelial cells (Figure 3B). SMA staining served as a marker for paSMC localization (Figure 3C). At the single-cell level, RACK1 and BMPRII exhibited regions of colocalization within the cytoplasm and at the cell surface, as demonstrated by immunofluorescence costaining of BMPRII and RACK1 (Figure 3D through 3F).

Figure 1. Identification of RACK1 as novel interaction partner for BMPRII using the yeast 2-hybrid system. A, Schematic representation of the 3 library clones, clones 42, 58, and 97, identified by yeast 2-hybrid screen encoding interaction partners for BMPRII, and full-length RACK1, in which the positions of the 7 WD domains of RACK1 are indicated. Numbers of amino acid residues are indicated. B, Yeast 2-hybrid interactions between the full-length wild-type RACK1 or truncated RACK1 variants encoded by clones 42, 58, and 97, fused to the AD of the GAL4 transcriptional regulator, with the full-length intracellular domain (BD-BMPRII total) of BMPRII or its kinase domain (BD-BMPRII kinase) fused to the DNA-binding domain (BD) of the GAL4 transcriptional regulator. The AD and BD expressed separately served as a negative control.

RACK1-BMP Receptor Expression in paSMCs

To quantitatively analyze the expression of RACK1 and BMP receptors in paSMCs, we performed real-time RT-PCR of RACK1 and all BMP receptors using mRNA derived from paSMCs cultured from lobar pulmonary arteries of control and IPAH patients exhibiting BMPR2 mutations.26 We detected a significant reduction in BMPRII but not BMPRIA, BMPRIB, or RACK1 expression levels in paSMCs derived from IPAH patients (Figure 4G). Of note, most of the BMPRII expressed in paSMCs can be attributed to the full-length molecule, whereas expression of the short isoform is significantly lower, as detected using isoform-specific primers (Figure 4G).

RACK1 Expression in Monocrotaline-Induced Pulmonary Hypertension

To further explore the regulation of RACK1 in a nongenetic model of pulmonary hypertension, we chose the rat model of monocrotaline-induced PH. Using semiquantitative and quantitative RT-PCR, we found that the expression of RACK1 mRNA was significantly downregulated 4 weeks after monocrotaline administration compared with control rats or rats 2 weeks after monocrotaline administration (Figure 5A and 5B). Similarly, RACK1 protein expression was significantly downregulated after 4 weeks but not 2 weeks of monocrotaline administration, as depicted by Western blot analysis and densitometry (Figure 5C and 5D). The reduced BMPRII expression also was observed in this model29 further argues for a dramatic reduction of RACK1–BMPRII interaction and an effect thereof on PAH pathogenesis.
Figure 2. Interaction of wild-type and mutated BMPRII constructs with RACK1 in mammalian cells in vivo. A, Interaction of GST-tagged BMPRII with Myc-tagged RACK1. Myc-tagged RACK1 was associated with glutathione-sepharose in the presence of GST-tagged BMPRII (lane 1, top blot) but not in the presence of GST alone (lane 2, top blot). B, Coimmunoprecipitation of HA-tagged BMPRII and Myc-tagged RACK1 in a ligand-independent manner. NIH-3T3 cells were transiently transfected with a plasmid encoding BMPRII-HA (lanes 3 and 4) and Myc-RACK1 (lanes 2 through 4). Myc-tagged RACK1 was immunoprecipitated from cell lysates with an anti-Myc antibody, and immunoprecipitates were probed with an anti-HA antibody to detect BMPRII. HA-tagged BMPRII was detected in RACK1 immunoprecipitates prepared with an anti-Myc antibody, regardless of BMP stimulation (lanes 3 and 4, top blot). Proper and equal protein expression is illustrated in the middle and lower blots. Lysates from untransfected NIH cells served as a negative control (lane 1). C, Overexpressed BMPRII variants carrying amino acid substitutions were visualized by Coomassie Blue staining. D, GST pull-down of Myc-tagged RACK1 using the indicated GST-tagged BMPRII variants. Pull-down of Myc-tagged RACK1 is illustrated in the top; proper expression and equivalent input of Myc-tagged RACK1 are illustrated in the bottom. Band intensities were quantified by densitometry and are illustrated in the histogram underneath the blots. *P<0.05. E, Evaluation of plasmid transfection into human paSMCs. Either empty vector (EV) or an EGFP expression vector was transfected into primary paSMCs and cultured for 24 hours. Cells were then trypsinized and analyzed for EGFP expression by FACS. F, Synchronized paSMCs transfected with cDNA encoding wild-type BMPRII or a truncated BMPRII variant as a result of a premature stop codon (R332X) were harvested after 24 hours, fixed, stained, and analyzed for DNA content by flow cytometry. Percentages of cells in the G0/G1 phase (green), S phase (pink), and G2/M phase (blue) are indicated. Cells transfected with EV served as a negative control for the assay.
Functional Effects of Alterations in RACK1 Expression on paSMC Proliferation

Because paSMC proliferation is a key event in the development of PAH and because RACK1 expression was significantly altered in PAH, we continued by investigating the effect of RACK1 knockdown, observed in the monocrotaline model, on paSMC proliferation. For this purpose, we initially designed 4 different siRNAs directed against RACK1. Using this approach, we were able to knock down RACK1 expression by 72%, as assessed by real-time RT-PCR and Western blot analysis (Figure 6A and 6B). As depicted in Figure 6C, RACK1 knockdown by siRNA treatment resulted in a significan-

![Figure 3. Localization of BMPRII and RACK1 in healthy lungs and isolated paSMCs. A through C, Immunohistochemical analysis of endogenous BMPRII (A), RACK1 (B), and SMA (C) expression in the normal human lung. D through F, Immunofluorescence analysis of BMPRII and RACK1 expression in primary human paSMCs. BMPRII was visualized with an FITC-labeled antibody (green; D); RACK1 was visualized with an Alexa 594-labeled antibody (red; E). Colocalization results in a yellow cellular staining (F). DAPI staining (blue) facilitated visualization of the nuclear compartment of the cells.]

![Figure 4. Localization of BMPRII and RACK1 in lungs of patients with IPAH. Localization of BMPRII (A and B), RACK1 (C and D), and SMA (E and F) was assessed in large vessels (left column) and in plexiform lesions (right column). The mRNA expression of BMP receptors, including total BMPRII, its long form (LF), its short form (SF), BMPRIA, BMPRIB, or RACK1, was analyzed by real-time PCR (data reflect the mean±SEM). *P<0.05.]

icant increase in paSMC proliferation compared with mock siRNA-transfected cells. These data further support a role for RACK1 in the regulation of paSMC proliferation and suggest that perturbations to RACK1 expression and/or function may lead to enhanced paSMC cell growth. Next, the effect of RACK1 overexpression on paSMC proliferation was assessed by cell cycle analysis and [3H]-thymidine incorporation. Cell cycle analysis demonstrated that overexpression of RACK1 led to an arrest of cell proliferation, as evident by an increase in the G0/G1 population, and a decrease in the number of cells in the S and G2/M phases (Figure 7A). Importantly, thymidine incorporation revealed a >50% decrease in paSMC proliferation compared with cells transfected with empty vector (Figure 7B). Platelet-derived growth factor was used as a positive control.

### Effect of RACK1 on BMP Signaling

Finally, we investigated the effect of modulating RACK1 expression on Smad signaling by analyzing BMP2-dependent Smad1 phosphorylation under conditions of RACK1 overexpression or knockdown (Figure 8A and 8B). Although RACK1 overexpression led to increased Smad1 phosphorylation, RACK1 downregulation resulted in reduced Smad1 phosphorylation. In agreement with this, RACK1 overexpression also induced a 2-fold increase in pId120 luciferase expression after BMP2 stimulation compared with cells transfected with empty pcDNA vector (Figure 8C).

### Discussion

The present study tested the hypothesis that novel interaction partners of BMPRII are essential regulators of SMC proliferation, a key finding in PAH. The basis of this hypothesis is the observation that up to 50% of patients with familial PAH exhibit germ-line mutations in \( \text{BMPR2} \), the gene locus encoding BMPRII. Despite this overwhelming genetic evidence for a causal involvement of \( \text{BMPR2} \) mutations in PAH pathogenesis, we are still unclear about the precise mechanisms that give rise to such a localized disease in the setting of a germ-line mutation, which leads to the expression of the BMPRII protein variants throughout the body. Because most mutations described thus far are localized to the kinase domain and predicted to lead to a truncation of BMPRII protein, a loss and/or gain of function resulting from the specific mutations is proposed to be involved in PAH pathogenesis. Indeed, several recent publications have indicated that \( \text{BMPR2} \) mutations can alter intracellular signaling by p38 kinase or Smad proteins, although considerable discussion exists as to whether BMPRII mutations or its allelic loss increases or decreases Smad signaling.

Furthermore, truncations of BMPRII could result in the gain/loss of interaction of an as-yet unknown binding partner of BMPRII in a ligand-dependent or -independent manner. Therefore, the aim of our study was to uncover novel BMPRII-interacting proteins, to elucidate their function in...
paSMCs, and to investigate their localization and expression in healthy and diseased lungs. Here, we have identified RACK1 as a novel interaction partner of BMPRII using the yeast 2-hybrid system. This novel interaction was confirmed by GST pull-down and coimmunoprecipitation in mammalian cells and occurred in a BMP2-independent manner, suggesting a constitutive interaction involved in basal maintenance of the smooth muscle phenotype.

**Figure 6.** Effect of RACK1 depletion on proliferation of primary paSMCs. A, RNA levels of RACK1 in paSMCs transfected with 4 different siRNA (si 1 through 4) were assessed by quantitative RT-PCR. Expression differences between siRNA- and mock-treated cells are depicted as log fold-change (ΔΔCt) values. B, Western blot of protein extracts obtained from paSMCs after siRNA treatment. β-Actin served as a loading control. C, Proliferation of paSMCs after transfection with siRNA against RACK1 was assessed by [3H]-thymidine incorporation. Values represent the mean±SEM of 3 independent experiments. *P<0.05.

**Figure 7.** Effect of RACK1 overexpression on proliferation of primary human paSMCs. A, Synchronized paSMCs transfected with empty vector (EV) or a RACK1-expressing vector were harvested after 24 hours, fixed, stained, and analyzed for DNA content by flow cytometry. Percentages of cells in the G0/G1 phase (green), S phase (pink), and G2/M phase (blue) are indicated. B, paSMC proliferation was assessed by direct [3H]-thymidine incorporation analysis (n=3). Platelet-derived growth factor (PDGF) stimulation was used as a positive control for the assay. *P<0.05.
RACK1 is a 36-kDa cytosolic protein that is composed of 7 WD40 motifs, which are predicted to form a 7-bladed propeller structure important in protein-protein interactions. These WD repeats are highly conserved among species, including plants, Drosophila melanogaster, higher mammals, and humans. RACK1 is expressed ubiquitously in most tissues such as brain, heart, kidney, liver, pancreas, spleen, or lung, suggesting an important homeostatic function in different cell types. RACK1 was originally identified on the basis of its ability to bind the activated form of protein kinase C, described to stabilize the active form of protein kinase C, and to facilitate its protein trafficking within the cell. RACK1 binds to and inhibits Src family kinases, which also were recently described to interact with BMPRII. Through its interaction with protein kinase C or Src kinases, RACK1 can function as a critical adaptor protein mediating cross-talk between serine/threonine and tyrosine kinase signaling pathways. In addition, RACK1 has been described to interact with integrins, the common β chain of the interleukin-5/interleukin-3/granulocyte macrophage colony-stimulating factor receptor, Src, β-spectrin and dynamin, protein tyrosine phosphates PTP, and PDE4D5. These reports suggest that RACK1 acts as a scaffold providing the platform for protein-protein interactions essential in the recruitment of its binding partners to transmembrane receptors.

RACK1 interacts with its binding partners in 2 ways in a constitutive fashion such as with the cAMP-specific phosphodiesterase PDE4D5 or in a stimulus-dependent fashion such as with protein kinase C isoforms. The RACK1–BMPRII interaction reported here represents a constitutive interaction in that coimmunoprecipitation experiments have not shown any influence of BMP-2 stimulation on this interaction. In addition, RACK1 has been described to be able to directly interact with 1 specific protein at a time, offering the intriguing possibility of alternative signaling in the presence and/or absence of functional BMPRII such as in patients with BMPR2 mutations.

In light of the BMPR2 mutations that occur in IPAH patients, we determined whether BMPRII truncations affected their interaction with RACK1. To do so, we genetically engineered 4 different nonsense mutations derived from IPAH families, which generated a premature stop codon within the BMPR2 cDNA at position 1483, 1397, 1348, or 994, respectively. All of these mutants led to the expression of a truncated receptor that lacked the long intracellular tail, along with different stretches of the BMPRII kinase domain. Expression constructs encoding the shortest of these truncated BMPRII variants led to increased cell proliferation, as measured by cell cycle analysis, when transfected into primary paSMCs. In contrast, wild-type BMPRII led to decreased paSMC proliferation.
rendering further support to an antiproliferative role of BMPRII, even in the absence of ligand. These findings suggest that mutations of BMPRII do not completely prevent RACK1–BMPRII interaction, but the decreased intensity of BMPRII/RACK1 interaction may affect downstream signaling and cell proliferation and finally contribute to the pathogenesis of PAH. We also conclude that RACK1 requires the full-length kinase domain of BMPRII for efficient interaction, whereas the long intracellular tail encoded by exon 13 does not influence its binding.

To elucidate the potential in vivo relevance of the findings described thus far, we have performed immunohistochemical analyses of RACK1 and BMPRII localization in normal and PAH lungs, as well as in primary human paSMCs. The data derived from these studies indicated that BMPRII and RACK1 are coexpressed in paSMCs of pulmonary arteries and arterioles, the key sites of the vascular remodeling process observed in PAH. This clearly adds weight to the potential impact of RACK1 and its interaction with BMPRII in regulating essential paSMC functions such as proliferation, contraction, or secretion of extracellular matrix components. Further support for a pathogenic role of RACK1 in PAH was rendered by our expression studies in an animal model of PAH, the monocrotaline-induced rat model of PAH. These studies have found that RACK1 mRNA and protein levels were significantly decreased 4 weeks after monocrotaline administration (Figure 4), at a time when pulmonary vascular remodeling occurred. 

Interestingly, we did not observe any expression changes in RACK1 in paSMCs isolated from IPAH patients with BMPR2 mutations (Figure 4G). These analyses, however, have unraveled the downregulation of BMPRII in IPAH paSMCs and underscored the high expression levels of RACK1 in paSMCs.

Abrogation of RACK1 expression by siRNA treatment resulted in significantly increased paSMC proliferation (Figure 6), whereas overexpression of RACK1 decreased paSMC proliferation (Figure 7). Reduced RACK1 expression also led to decreased Smad signaling, whereas forced overexpression of RACK1 augmented Smad signaling (Figure 8), which is in line with the antiproliferative function ascribed to BMP-Smad signaling in paSMCs. Taken together, these functional data derived from primary pulmonary paSMCs clearly point toward a key regulatory role of RACK1 in paSMC growth.

We essentially hypothesize that a loss of interaction between BMPRII and RACK1 results in less BMP signaling and hence a loss of the antiproliferative effect of BMP on paSMCs. The net result is increased proliferation. In humans but not in the animal model, BMPRII truncations perturb the BMPRII-RACK1 interaction. The situation is different in the monocrotaline model because RACK1 levels are reduced. However, BMPRII levels also are reduced; thus, it is reasonable to speculate that the level of interaction between BMPRII and RACK1 also would be affected. In sum, although RACK1 levels are affected differently in humans and the monocrotaline model, the RACK1–BMPRII interaction would be impaired in both pathologies, and in both instances, we would expect to see enhanced proliferation as a consequence of this impaired RACK1–BMPRII interaction. Indeed, this is what is observed. Although BMP ligands generally exhibit antiproliferative properties on paSMCs, this effect is lost in paSMCs from patients with IPAH who harbor mutations in the gene encoding BMPR2. Similarly, we also have demonstrated that paSMCs from monocrotaline-treated rats, which exhibited reduced BMPRII levels, are insensitive to the antiproliferative effects of BMP ligands. Thus, our data indicate that loss of interaction between BMPRII and RACK1 results in less BMP signaling and hence loss of the antiproliferative effect of BMP on paSMCs and finally increased paSMC proliferation. Although we would hesitate to propose that this is a dominant mechanism in all PAH patients, our data suggest that this may contribute to PAH pathogenesis in those patients with BMPR2 mutations that lead to a truncation and/or loss of its kinase domain.

Conclusions

Genetic associations and functional genomics strongly point to BMPR2 mutations as a causal factor in PAH. In addition, genetic, somatic, and/or environmental factors such as appetite suppressants, hypoxia, survivin, or serotonin undoubtedly also play a role in the pathobiology of PAH. In recent years, BMPRII function was studied intensively, and with the yeast 2-hybrid assay, 3 novel interaction partners have been reported. Tctex 1, a light chain of dynein, was identified as a novel BMPRII interacting molecule. This molecule binds to and is phosphorylated by BMPRII and was suggested to interact with downstream mediators of BMPRII (eg, Smads). Src kinase was reported as another BMPRII interaction partner. The interaction between BMPRII and Src inhibited Src kinase activity by reducing its phosphorylation at tyrosine 418 in a ligand-dependent manner and further inhibited downstream cell cycle regulators suggested to influence cell proliferation. In addition, BMPRII was identified in a recent screen for LIM kinase–interacting proteins, all clones identified as encompassing the tail region of BMPRII. Further analyses revealed that the interaction between LIMK1 and BMPRII inhibited the ability of LIMK1 to phosphorylate cofilin, which was alleviated by the addition of BMP4. Most interestingly, LIMK1-BMPRII interaction altered the actin cytoskeleton dynamics. Although these investigations have led to an increased understanding of BMPRII functions, we are now at a stage where novel BMPRII interaction partners play a role in pathological SMC functions such as cell proliferation with direct relevance to disease.

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References


Disclosures

None.
CLINICAL PERSPECTIVE

Pulmonary arterial hypertension (PAH) is a fatal disease characterized by elevated blood pressure in the pulmonary circulation resulting from a progressive increase in pulmonary vascular resistance. Pathogenic mechanisms of PAH include vasoconstriction, in situ thrombosis, and remodeling of the pulmonary arterial vessel wall that results in medial hypertrophy as a consequence of augmented proliferation of endothelial and pulmonary artery smooth muscle cells. Patients suffering from familial PAH exhibit heterogeneous germ-line mutations in the BMPR2 locus, a gene encoding the type II bone morphogenetic protein receptor (BMPRII). Although genetic studies have assigned a causal role for BMPR2 mutations in the onset and/or development of PAH, our knowledge of the functional contribution of these mutations is still evolving, and PAH remains an incompletely understood and essentially incurable disease. In the present study, we have identified the receptor of activated C-kinase (RACK)-1 as a novel interaction partner of BMPRII using a 2-hybrid screen. RACK1 colocalized with BMPRII in pulmonary arterial smooth muscle cells in vitro and in vivo and was a negative regulator of pulmonary arterial smooth muscle cell proliferation. Therefore, our studies have identified a novel signaling intermediate downstream of BMPRII, which essentially controls pulmonary arterial smooth muscle cell proliferation. Modifying RACK1 expression or enhancing RACK1–BMPRII interaction represents a novel option in reverse vascular remodeling, a therapeutic goal for patients with PAH.
Receptor for Activated C-Kinase 1, a Novel Interaction Partner of Type II Bone Morphogenetic Protein Receptor, Regulates Smooth Muscle Cell Proliferation in Pulmonary Arterial Hypertension

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