CIB1 Regulates Endothelial Cells and Ischemia-Induced Pathological and Adaptive Angiogenesis

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Abstract—Pathological angiogenesis contributes to various ocular, malignant, and inflammatory disorders, emphasizing the need to understand this process on a molecular level. CIB1 (calcium- and integrin-binding protein), a 22-kDa EF-hand–containing protein, modulates the activity of p21-activated kinase 1 in fibroblasts. Because p21-activated kinase 1 also contributes to endothelial cell function, we hypothesized that CIB1 may have a role in angiogenesis. We found that endothelial cells depleted of CIB1 by either short hairpin RNA or homologous recombination have reduced migration, proliferation, and tubule formation. Moreover, loss of CIB1 in these cells decreases p21-activated kinase 1 activation, downstream extracellular signal-regulated kinase 1/2 activation, and matrix metalloproteinase 2 expression, all of which are known to contribute to angiogenesis. Consistent with these findings, tissues derived from CIB1-deficient (CIB1−/−) mice have reduced growth factor–induced microvessel sprouting in ex vivo organ cultures and in vivo Matrigel plugs. Furthermore, in response to ischemia, CIB1−/− mice demonstrate decreased pathological retinal and adaptive hindlimb angiogenesis. Ischemic CIB1−/− hindlimbs also demonstrate increased tissue damage and significantly reduced p21-activated kinase 1 activation. These data therefore reveal a critical role for CIB1 in ischemia-induced pathological and adaptive angiogenesis. (Circ Res. 2007;101:1185-1193.)

Key Words: angiogenesis ■ endothelial cells ■ ischemia ■ CIB1 ■ PAK1

Ischemia-induced angiogenesis involves both pathological and adaptive angiogenesis and has a clear association with many disorders that involve various types of human tissue.1 In ischemic retinal diseases, such as diabetic retinopathy, retinopathy of prematurity, and age-related macular degeneration, angiogenesis is excessive; the goal in these retinal diseases is to inhibit ischemia-induced pathological angiogenesis.2–4 Alternatively, in ischemic diseases, such as peripheral vascular disease and ischemic heart disease, the induction of adaptive angiogenesis is beneficial in preventing tissue damage and promoting recovery.5 Central to this process are vascular endothelial cells (ECs), which respond to both anti- and proangiogenic factors and on activation, undergo a remarkable increase in migration, proliferation, nascent tubule formation, and matrix metalloproteinase (MMP) secretion.6,7 Identifying exactly which molecules contribute to these processes, but not to physiological angiogenesis (angiogenesis during development and tissue regeneration), will provide better therapeutic targets to alleviate ischemia-associated diseases and aid in our understanding of how pathological angiogenesis arises.

CIB1 (also known as calmyrin or kinase-interacting protein [KIP]) is a 22-kDa EF-hand–containing protein identified originally in a yeast 2-hybrid screen as a binding partner for the cytoplasmic tail of the platelet integrin αIIb.8 It was later determined that CIB1 inhibits agonist-induced αIIbβ3 activation in megakaryocytes.9 However, CIB1 is widely distributed, suggesting that it has cellular functions independent of αIIbβ3.10 Accordingly, CIB1 appears to regulate various cellular processes by associating with proteins such as the Alzheimer’s disease-associated presenilin-2,11 the inositol 1,4,5-triphosphate receptor,12 focal adhesion kinase (FAK),13 and p21-activated kinase (PAK1).14 Among these binding partners, FAK and PAK1 are known to regulate EC function and in vivo angiogenesis.15,16

We recently engineered a CIB1-deficient (CIB1−/−) mouse via homologous recombination in embryonic stem cells. Although CIB1−/− mice reach all developmental milestones, adult male mice are sterile because of a defect in the haploid phase of spermatogenesis.17 Here we demonstrate that loss of CIB1 attenuates PAK1 and extracellular signal-regulated kinase (ERK)1/2 activation and MMP-2 expression. We also demonstrate that CIB1 is critical for various EC functions and that CIB1−/− mice have significant defects in growth factor- and ischemia-induced pathological and adaptive angiogenesis. Therefore, our studies identify CIB1 as a target for therapy in angiogenesis-dependent clinical disorders.
Materials and Methods

Reagents, Cells, and Short Hairpin RNA Construction

All reagents, cell types, blotting techniques, and short hairpin (sh)RNA construction are described in the online data supplement at http://circres.ahajournals.org.

Boydner Chamber Haptotactic, Matrigel Tubule Formation, and ELISA Assays

Haptotactic migration and tubule formation were performed as described previously with minor modifications. These assays and ELISA proliferation assays are described in the online data supplement.

Gelatin Zymography

The activity of MMP-2 in conditioned media was determined by gelatin zymography. Briefly, ECs were serum starved for at least 24 hours, followed by addition of phenol-free basal medium supplemented with 100 ng/mL of growth factor. After 18 hours, conditioned media were collected, filtered through 0.45-μm Whatman filters, and concentrated using a Vivaspin 10-kDa concentrator (Sartorius, Edgewood, NY). Equal protein concentrations were loaded under nonreducing conditions onto premaid 10% zymograms and developed according to the instructions of the manufacturer (Bio-Rad, Hercules, Calif). For each experiment, n=3.

Ex Vivo Tissue Cultures

Tissue culturing was performed as described previously with minor modifications. Wild-type (WT) and CIB1−/− aortic rings were embedded between 2 layers of 250 μL of growth factor-reduced Matrigel in a 24-well culture format and cultured in 300 μL of growth media or basal media supplemented with 50 ng/mL basic fibroblast growth factor (bFGF) or vascular endothelial growth factor (VEGF). After 2, 5, and 8 days, images of aortic rings were collected and stitched together using PhotoFit Premium software (Tekmate, Tokyo, Japan). At each time point, the number of sprouting vessels were counted using ImageJ as described previously (n=8 per group). Identical procedures were used for the tibialis anterior muscle cultures and were analyzed in a similar manner after 8 days of culturing.

In Vivo Matrigel Plug Assay

Matrigel plug assays were performed as described previously with minor modifications. WT and CIB1−/− mice were subcutaneously injected with ~300 μL of growth factor-reduced Matrigel mixed with 60 U/mL heparin or with heparin plus 250 ng/mL bFGF or VEGF. After 14 days, plugs were isolated along with adjacent skin and periosteal muscle, fixed, and sectioned at interrupted intervals. Blood vessel infiltration was analyzed in 4 random hematoxylin/eosin (H&E)-stained interrupted sections using a Nikon inverted microscope and expressed as mean values of infiltrating blood vessels per plug (n=3 per group).

Oxygen-Induced Retinal Angiogenesis Assay

Postnatal day 7 (P7) mice were placed in 75% oxygen for 5 days. Following hyperoxic incubation, P12 CIB1−/− and WT neonatal mice were housed for 5 additional days under normoxic conditions, after which P17 mouse eyeballs were fixed in 2% paraformaldehyde and either microdissected to obtain retinal flat mounts or cryopreserved in OCT (IMEB, San Marcos, Calif). Fluorescent images of flat mounts stained with Alexa594-conjugated Griffonia simplicifolia isoelectin B4 (isolectin GSL-1-B4; 1:100, Invitrogen) were analyzed with a Nikon inverted microscope (PhotoFit, TekMate, Anchorage, Alaska; ImageJ; n=8 mice per group). Interrupted 6-μm sections at 50-μm intervals were obtained for cryopreserved eyeballs and H&E stained. Mean capillary area of vessels either directly under or invading the retinal inner limiting membrane (ILM) were measured from digitized images obtained from 2 randomly selected interrupted eyeball sections (n=4 mice per group).

Unilateral Femoral Artery Ligation, Laser Doppler Perfusion Imaging, and Appearance and Use Score Assessments

Ligation, imaging, and use scores were performed as described previously. For each sample, 5 sections (50 μm apart) were obtained and stained with H&E or Masson’s trichrome or labeled with isolectin GSL-1-B4 (1:100). Three 20× images were collected for each condition from 2 randomly selected sections. Using ImageJ, muscle atrophy and capillary density were estimated as described previously (n=5–9 mice per group).

Atrophy and Capillary Density in Gastrocnemius Muscles

Twenty-one days after femoral artery ligation, gastrocnemius muscles were fixed and paraffin embedded as described previously. For each sample, 5 sections (50 μm apart) were obtained and stained with H&E or Masson’s trichrome or labeled with isolectin GSL-1-B4 (1:100). Three 20× images were collected for each condition from 2 randomly selected sections. Using ImageJ, muscle atrophy and capillary density were estimated as described previously (n=5–9 mice per group).

Animal Regulations

All housing, breeding, and experimental procedures with mice were in accordance with national guidelines and regulations, and were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

Statistical Analysis

Continuous variables were compared with either the Student’s t test or nonparametric Mann–Whitney U test. We considered P<0.05 to be significant. For animal studies, male and female mice demonstrated no experimental differences; thus they were combined.

Results

Loss of CIB1 Disrupts Normal EC Function

CIB1 is expressed in highly vascularized adult mouse organs, embryonic vascular structures, and ECs (Figure I in the online data supplement). Because CIB1 associates with angiogenic kinases such as PAK1 and FAK, we hypothesized that CIB1 may contribute to EC function. We first tested the role of CIB1 in EC migration using a conventional Boydner chamber haptotactic migration assay. Knockdown of CIB1 with shRNA (supplemental Figures II and III) decreased haptotaxis of immortalized mouse embryonic heart ECs (MECs) on fibronectin (38% relative to control MECs; Figure 1A). Similar results were also obtained in the presence of actinomycin D, demonstrating that the haptotactic assay was not significantly influenced by EC proliferation during the relatively short period of this assay (data not shown). Similarly, mouse lung ECs (MLECs) isolated from CIB1−/− mice demonstrated a similar decrease in migration (24% decrease relative to WT and CIB1−/− MLEC; Figure 1B). Although angiogenic growth factors bFGF and VEGF increased haptotaxis in untransduced MECs (data not shown) and control transduced MECs, they did not rescue haptotaxis in CIB1 knockdown MECs (Figure 1A). Interestingly, reintroduction of CIB1 in CIB1−/− MLEC restored haptotactic migration up
to, but not beyond, normal levels (Figure 1B). To confirm these findings, we also tested the role of CIB1 in EC migration using a monolayer wound-healing assay. Monolayer wounds in both CIB1-depleted MECs and CIB1−/− mouse heart ECs (MHECs) resolved less rapidly, relative to control transduced MECs and WT MHECs, respectively (supplemental Figure IV). Therefore, these data demonstrate that CIB1 expression is necessary for normal EC migration.

Proliferation of ECs is also fundamental to angiogenesis.6 Relative to control, MECs depleted of CIB1 by shRNA and cultured in either growth media or basal media supplemented with either bFGF or VEGF demonstrated modest yet significantly decreased 5-bromodeoxyuridine (BrdUrd) incorporation (Figure 1C). No difference in proliferation between untransduced and control transduced MECs was observed (data not shown). Consistent with this, CIB1−/− MHECs and MLECs cultured in growth media also demonstrated a 61% and 35% decrease, respectively, in BrdUrd incorporation compared with WT ECs (Figure 1D). Direct cell counts of cultured CIB1-depleted MECs and CIB1−/− MHECs further confirmed a significant decrease in proliferation relative to controls (data not shown). Thus, in addition to migration, CIB1 is also necessary for normal rates of EC proliferation.

To determine whether loss of CIB1 affects other EC functions, we also assessed tubule formation on growth factor–reduced Matrigel. In the presence of growth media, CIB1-depleted MECs and CIB1−/− MHECs showed a 26% and 44% reduction in nascent tubule formation compared with their respective controls (Figure 1E and 1F). Treatment of CIB1-depleted MECs and CIB1−/− MHECs with bFGF or VEGF did not stimulate tubule formation to the same extent as control cells (Figure 1E and 1F). Hence, in addition to EC migration and proliferation, CIB1 appears to be required for efficient EC sprouting and invasion, events required for angiogenesis.

Loss of CIB1 in ECs Disrupts PAK1 and ERK1/2 Activation

We previously determined that CIB1 regulates PAK1 activation in fibroblasts and transformed epithelial cells.14 Naik and Naik also have reported that CIB1 can regulate FAK activation.13 Because several studies have underscored the importance of PAK1, FAK, and their downstream mitogen-activated protein kinase (MAPK) effectors, ERK1/2 and p38, in EC angiogenic signaling,15,16,24 we asked whether loss of CIB1 affects the activation of these signaling molecules. Although FAK phosphorylation was not significantly altered in CIB1−/− ECs (data not shown), in vitro kinase assays revealed significantly less active PAK1 in CIB1−/− MLECs relative to control cells, 20 and 45 minutes following adhesion to fibronectin (Figure 2A). Decreased PAK1 activation was also observed in CIB1-depleted MECs (data not shown).
Similarly, decreased ERK1/2 phosphorylation was observed in CIB1+/− MLECs, without changes in total ERK1/2 protein levels, 20 and 45 minutes following adhesion (Figure 2B). Control ECs also showed differences in the relative peak activation times of PAK1 and ERK1/2, suggesting the contribution of additional signaling pathways leading to ERK1/2 activation. However, despite these differences, we consistently found decreased activation of both PAK1 and ERK1/2 in CIB1+/− ECs. Importantly, no differences in protein kinase C and p38 MAPK phosphorylation were detected in WT and CIB1+/− MLECs following adhesion (Figure 2B and data not shown). Thus, these data demonstrate that loss of CIB1 can disrupt EC signaling, potentially via regulation of PAK1 and ERK1/2 activation in ECs.

**Loss of CIB1 in ECs Decreases MMP-2 Expression**

Numerous signaling cascades can be activated downstream of PAK1. Among them are MAPKs that are implicated in the production and activation of MMPs. Because loss of CIB1 in ECs resulted in decreased responsiveness to angiogenic growth factors and decreased PAK1 and ERK1/2 activation on adhesion to fibronectin, we asked whether MMP expression was also affected. Enzymatic activity of MMPs was assessed by gelatin zymography, which revealed increased lytic zones only at the molecular mass of MMP-2 (68 kDa) in the conditioned media of growth factor–treated WT MLECs but not CIB1+/− MLECs (Figure 2C). Western blotting also demonstrated less growth factor–induced MMP-2 expression in CIB1+/− MLEC conditioned media (Figure 2D), and decreased MMP-2 protein expression in lysates derived from CIB1+/− MHECs (Figure 2E). However, MMP-9 secretion was undetectable in WT and CIB1+/− ECs (data not shown). Furthermore, WT and CIB1+/− ECs showed no differences in the expression of the physiological inhibitor (tissue inhibitor of metalloproteinases [TIMP]-2) and activator of MMP-2, (MMP-14 [MT1-MMP]) (data not shown).

**CIB1 Deficiency Diminishes bFGF and VEGF-Induced Microvessel Growth**

The decreased response to bFGF and VEGF and attenuated MMP-2 expression by CIB1+/− ECs led us to ask whether angiogenesis is decreased ex vivo, in cultured microsections of aortas and tibialis anterior muscles from CIB1+/− mice. CIB1+/− aortic rings and tibialis anterior muscles cultured for 8 days in growth media produced ≈50% fewer microvessels, relative to control (Figure 3A through 3C). In addition, CIB1+/− aortic rings also produced ≈60% fewer microvessels in response to bFGF and VEGF (Figure 3A and 3B). We further assessed growth factor–induced angiogenesis in vivo by implanting WT and CIB1+/− mice with Matrigel plugs impregnated with bFGF or VEGF. Microvessel infiltration into the plugs was reduced by ≈80% in CIB1+/− mice compared with WT littermates (Figure 3D and 3E). Thus, growth factor–induced angiogenesis is strongly inhibited in CIB1+/− mice.

**CIB1 Deficiency Leads to Decreased Oxygen-Induced Retinal Angiogenesis**

Mice lacking CIB1 develop and reach adult reproductive age normally. Therefore, not surprisingly, we observed that developmental retinal angiogenesis in CIB1+/− mice proceeds normally (supplemental Figure V). However, because CIB1 appeared to be a critical regulator of EC function and signaling, we hypothesized that CIB1+/− mice may still have a defect in pathological retinal angiogenesis. To test this, we examined CIB1+/− mice using an oxygen-induced retinal angiogenesis assay, wherein in vivo exposure to an elevated inspired oxygen concentration obliterates the central retinal capillaries, leading to hypoxia in the mouse neonatal retina. Subsequent return to normoxia induces exuberant angiogenesis, resulting in formation of aberrant capillary neovessel buds that can be visualized in isolecitin GSL-1-B4–stained retinal flat mounts.

Well-defined buds were detected in WT P17 flat mounted retinas. In CIB1+/− retinas, fewer and less clearly defined buds were evident (Figure 4A and 4B). Because these buds are composed of clusters of tortuous vessels that protrude through the retinal ILM, retinal cross-sections were obtained to assess the size and morphology of these neovessels. WT retinal cross-sections displayed large protruding mi-

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**Figure 2. Loss of CIB1 disrupts PAK1 activation and MMP-2 expression.** A, PAK1 activity in WT and CIB1−/− MLECs held in suspension or adhered to fibronectin for the indicated times. PAK1 in vitro kinase assays were performed using myelin basic protein as substrate ([32P]MBP). Western blots of total PAK1 protein and Coomassie stain of myelin basic protein serve as loading controls. B, Thr514 protein kinase C (PKC) and ERK1/2 phosphorylation in WT and CIB1−/− MLECs held in suspension or adhered to fibronectin. Western blots of total ERK1/2 and Rac serve as loading controls. C, Conditioned media of growth factor–treated and untreated WT and CIB1−/− MLECs were subjected to gelatin zymography. Clear bands in destained gel represent MMP-2. D, Western blot of MMP-2 in conditioned media of growth factor–treated and untreated WT and CIB1−/− MLECs for C and D, Coomassie-stained nonspecific bands serve as loading controls. E, Western blot of total MMP-2 and CIB1 protein in duplicate WT and CIB1−/− MHEC lysates. Blot for GAPDH serves as a loading control. For each experiment, n=3.
CIB1 Deficiency Delays Postischemic Recovery and Neovascularization

A common mouse model for peripheral vascular disease is experimentally induced hindlimb ischemia through unilateral femoral artery ligation. In this model, blood flow to the hindlimb is effectively eliminated, which rapidly induces ischemia and postischemic recovery mechanisms (ie, adaptive angiogenesis and arteriogenesis). To extend our observations from the oxygen-induced retinal angiogenesis assay, we performed this assay to determine whether CIB1 also plays a role in adaptive ischemia-induced angiogenesis.

Noninvasive, laser-scanning Doppler velocimetry demonstrated that by 14 days postfemoral artery ligation, 90% of hindpaw plantar region perfusion was restored in WT mice but only 60% in CIB1−/− mice (Figure 5A and 5B). Furthermore, ischemic tissue injury was more severe in CIB1−/− mice, as assessed by appearance and use scores. Incidence of nail and toe discoloration, toe loss, hindpaw cyanosis, as well as impaired hindlimb function were several fold higher in CIB1−/− mice relative to WT littermates (Figure 5C and 5D). Similarly, increased atrophy and fibrosis were observed in the gastrocnemius muscles of CIB1−/− hindlimbs, which are known to experience substantial ischemia immediately after femoral artery ligation (Figure 6A). Average muscle fiber size in CIB1−/− ischemic gastrocnemius muscles was reduced by 23%, compared with only 6% in WT muscles (Figure 6B), indicating that CIB1−/− tissue experiences more profound tissue damage after ischemia.

Interestingly, tissue homogenates of CIB1−/− ischemic gastrocnemius muscles, isolated 4 days after femoral artery ligation showed a significant decrease in PAK1 activation and ERK1/2 phosphorylation (Figure 6C). PAK1/2 phosphorylation in CIB1−/− nonischemic gastrocnemius muscle tissue was also significantly reduced. However, no consistent differences were detected in p38 phosphorylation and MMP-2 expression between WT and CIB1−/− ischemic and nonischemic gastrocnemius muscles (Figure 6C). Thus, these data correlate with our findings in vitro and demonstrate that CIB1−/− mice have a specific defect in PAK1 and ERK1/2 signaling, which likely contributes to the decrease in recovery in CIB1−/− ischemic tissue.

Attenuated PAK1 activation may also lead to decreased angiogenesis, which could also explain the observed increase in tissue damage in CIB1−/− mice following femoral artery ligation. To test this, we determined capillary densities in microvessels that occasionally ruptured through the ILM, whereas CIB1−/− retinal cross-sections showed blood vessels directly under the ILM that were smaller in size and did not protrude through as frequently (Figure 4C and 4D). In CIB1−/− retinas, the ILM was also almost always intact (Figure 4C). Therefore, these data provide the first evidence demonstrating that CIB1−/− mice have impaired pathological angiogenesis in response to ischemia.
gastrocnemius muscles 21 days after femoral artery ligation in WT and CIB1−/− mice. Isolectin GSL-1-B4 staining revealed an increase in capillary density only in WT ischemic gastrocnemius muscles (Figure 6D and 6E), thus providing further evidence of reduced ischemia-induced angiogenesis in CIB1−/− mice. Because postmortem x-ray angiography revealed unaltered arteriogenesis in CIB1−/− mice (unpublished data, 2007), this defect in ischemia-induced angiogenesis appears to be specific.

**Discussion**

CIB1 is an important regulatory molecule that is expressed in different tissue types and has various binding partners. However, the role of CIB1 in ECs has never been explored. In this study, we use in vitro, ex vivo, and in vivo complementary analyses to provide the first evidence describing the critical role of CIB1 in EC function and signaling, growth factor–induced angiogenesis, and ischemia-induced pathological and adaptive angiogenesis.

**Figure 4.** Oxygen-induced retinal neovascularization is decreased in CIB1−/− mice. A, Isolectin GSL1–B4–stained flat mounted retinas from WT and CIB1−/− neonatal mice exposed to hyperoxic conditions followed by normoxia (left images). Vascular buds formed in WT and CIB1−/− ischemic retinas are indicated by arrowheads in magnified retinal areas (right images). Scale bar, 25 μm. B, Quantification of vascular buds per retina in WT and CIB1−/− mice (n=8 per mouse genotype). C, Representative H&E-stained cross-sections from WT and CIB1−/− retinas after exposure to hyperoxia, followed by normoxia. The WT retina shows microvessel protrusion (*) and rupture (arrow) through the ILM. Scale bar, 50 μm. D, Quantification of mean capillary area of microvessels directly under the ILM in WT and CIB1−/− retinas following ischemic injury (n=4 mice for each mouse genotype). V indicates vitreous; GCL, ganglion cell layer; IPL, inner plexiform layer. Error bars represent SEM. *P<0.05.

**Figure 5.** Reduced perfusion and recovery in CIB1−/− mouse ischemic hindpaws and hindlimbs following femoral artery ligation. A, Representative laser-scanning Doppler perfusion images of the ventral hindpaw surfaces of the same ischemic hindlimbs of a single WT and CIB1−/− mouse, performed in a blinded manner. Relative flow velocity is indicated by 6-hue pseudocolor, in which gray represents 0 and white represents maximal velocity (range of velocity values, 0 to 5000 perfusion units [PU], arbitrary units). Preligation relative blood flow velocity is high in both groups, as indicated by white and red. One day after ligation, blood flow is largely eliminated to the hindpaw, as indicated by blue. Evidence of toe gangrene is observed in CIB1−/− mouse ischemic hindpaws by 7 days. B, Quantification of perfusion at 7, 14, and 21 days after femoral artery ligation in WT and CIB1−/− mice (n=11 per genotype; *P<0.05, **P<0.001). C and D, Hindpaw appearance and hindlimb use scores were determined to assess rates of recovery in WT and CIB1−/− mice (n=11 per genotype). Mann–Whitney U test: *P<0.05, **P<0.01. Error bars represent ±SEM.
Recent evidence suggests that CIB1 contributes to important intracellular signaling mechanisms. For example, we previously demonstrated that CIB1 binds to and activates PAK1 in different cell types and that CIB1 depletion results in decreased PAK1 activation on adhesion to fibronectin. Furthermore, we previously observed a Cdc42-dependent upregulation of PAK1 activity in CIB1-depleted rat embryo fibroblasts with extended adhesion to fibronectin. In agreement with this, we also observed significantly reduced adhesion-induced PAK1 activation in CIB1−/− ECs. However, we did not detect a similar rebound of PAK1 activation with prolonged adhesion to fibronectin (up to 180 minutes) in CIB1−/− ECs, suggesting that this mechanism is either not as profound in ECs or occurs at later time points. More importantly, we found that PAK1 activation in vivo is dramatically reduced in CIB1−/− ischemic gastrocnemius muscle tissue homogenates. To our knowledge, this finding provides the first evidence demonstrating CIB1-dependent PAK1 activation in vivo and also suggests a role for PAK1 in ischemia-induced angiogenesis. Thus, these results reveal a functional interaction between CIB1 and PAK1 in multiple cell types and tissues.

Moreover, we previously demonstrated that CIB1 depletion increases fibroblast and epithelial cell migration. However, the present study demonstrates the opposite result, in which CIB1-depleted and CIB1−/− ECs exhibited decreased migration. These apparent differences may be attributable to a number of factors such as differences in cell types, activated signaling mechanisms, and/or experimental conditions. For example, the CIB1-binding partner PAK1 has been shown to both increase and decrease migration through different signaling pathways. In addition, experimental conditions used in this study were different than what was described previously (haptotactic migration in this study was toward 25 μg/mL fibronectin versus 5 μg/mL fibronectin), which may significantly affect the migratory response.

Previous reports also demonstrate that VEGF and bFGF can rapidly induce PAK activation, which in turn can phosphorylate and activate MEK1 and the MAPKs ERK1/2 and p38. Here we demonstrate that both PAK1 and ERK1/2 activation were significantly decreased in CIB1−/− ECs and CIB1−/− mouse nonischemic and ischemic gastrocnemius muscles. These defects appear to be specific because no profound differences were detected in the activation state of other kinases such as FAK, protein kinase C, and p38 MAPK. Moreover, the loss of CIB1 in part mimics the angiogenic defects observed when ERK1/2 is pharmacologically inhibited in vivo. Thus, it is likely that attenuated PAK1 and ERK1/2 activation contributes to the decrease in CIB1−/− ECs growth factor–induced functions, as well as microvessel formation in ex vivo CIB1−/− organ cultures and in vivo Matrigel plugs.

Additionally, ERK1/2 activation is necessary for transcriptional upregulation of MMPs, which are key modulators of pathophysiological events. Constitutively expressed by various cell types (including ECs), MMP-2 is known to contribute to pathological angiogenesis, including ischemia-induced retinal angiogenesis. Its contributions to angiogenesis are mediated by extracellular matrix degradation and proteolytic activation of other MMPs, such as proMMP-9, which is also essential in ischemia-induced angiogenesis. Accordingly, genetic deletion of MMP-2 and MMP-9 significantly reduces postnatal ischemia-induced angiogenesis. Here we report a similar phenotype in CIB1−/− mice and that CIB1−/− ECs generate less MMP-2. Thus, it is most likely that the defect in MMP-2 expression and secretion contributes to the blunted angiogenic response observed in CIB1−/− ischemic retinas and hindlimb gastrocnemius muscles.

Although CIB1−/− mice exhibit impaired ischemia-induced angiogenesis, they undergo normal developmental and physiological angiogenesis (developing retinal vasculature and female reproduction are normal). Although CIB1 homologs such as CIB2, CIB3, and CIB4 might compensate for the loss
of CIB1, it is more likely that CIB1 is a member of a growing group of genes that contributes to pathological angiogenesis during ischemia, inflammation, and tumor growth but not embryonic vasculogenesis and developmental angiogenesis. Prominent examples of these include knockout mice for bFGF, intercellular adhesion molecule 2, prostaglandin E2 receptor, and integrins β1 and β3. Collectively, our study identifies a previously uncharacterized role for CIB1 in the vasculature and for the first time demonstrates that it has a role in angiogenesis in vivo. Our study also demonstrates that CIB1 is critical in ischemia-induced pathological angiogenesis in the retina, as well as ischemia-induced adaptive angiogenesis in the hindlimb. However, CIB1 is not essential for developmental angiogenesis. Therefore, our studies identify CIB1 as a novel target for both pro- and antiangiogenic therapy.

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Disclosures

None.

References

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The paragraph titled "shRNA construction and transduction" has been corrected. The sequence originally labeled human CIB1 shRNA is now correctly labeled as mouse and mouse is now correctly labeled as human.

Expanded Materials and Methods

Reagents, lysate preparation, and Western blotting. MECs (obtained from Dr. R Auerbach, UW-Madison) and BAECs (obtained from Dr. C Patterson, UNC-Chapel Hill) were maintained in culture using high-glucose DMEM (Sigma, St. Louis, MO) supplemented with 3% fetal bovine serum (FBS; Sigma). HUVECs, HPAECs, and HCAECs were maintained in EBM culture media according to manufacture’s instructions (Cambrex, East Rutherford, NJ). MHECs and MLEC s were isolated in “isolation media” (high-glucose DMEM with 20% FBS, 20 U/mL penicillin/streptomycin antibiotic (Gibco, Carlsbad, CA), and 20 U/L Heparin (Sigma)) and were maintained in highly supplemented growth media (isolation media supplemented with 0.5X non-essential amino acids (Gibco), 0.5X sodium pyruvate (Gibco), 0.5X L-glutamate (Gibco), 25 mM HEPES (Cellgro, Lawrence, KS), and 100 µg/mL endothelial cell mitogen (ECGS; Biomedical Technologies, Stoughton, MA)). Cells were serum-starved in basal media (high-glucose DMEM supplemented with 0.1% BSA and 20 U/L heparin). For lysis of all EC types, 5 X 10^5 cells were pelleted and lysed in modified CHAPS buffer (20 mM Hepes, pH 7.4, 0.15 M NaCl, 10 mM CHAPS, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM each of CaCl₂ and MgCl₂, and Protease Inhibitors Cocktail Set III (Calbiochem, San Diego, CA)) on ice for at least 30 minutes, and lysates were collected following centrifugation. Total protein samples were separated by SDS-PAGE, transferred to a PVDF membrane, and subjected to Western blotting. Proteins were detected with CIB1 chicken polyclonal antibody, NH₂-terminal-specific anti-PAK1 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-Rac
polyclonal antibody (Cell Signaling, Inc., Boston, MA), anti-GAPDH polyclonal antibody (Santa Cruz Biotechnology, Inc.), pan γ thr514 anti-phospho-PKC polyclonal antibody (Cell Signaling, Inc.), and anti-total and anti-phospho-ERK1/2 polyclonal antibodies (Cell Signaling, Inc.). Total MMP-2 was detected using anti-MMP-2 specific goat IgG primary antibody (R&D Systems, Minneapolis, MN), and goat IgG secondary antibody (Sigma).

**Northern blotting.** Nitrocellulose blots of total adult mouse organ RNA and embryonic RNA were a generous gift from James Ferguson and Cam Patterson, UNC-CH. A full-length CIB1 probe was constructed using Prime It Random kit (Stratagene, La Jolla, CA). A CIB1 cDNA probe was labeled with 32P-dCTP and hybridized to nitrocellulose blots.

**shRNA construction and transduction.** A lentiviral expression system was adopted as previously described. For CIB1 knockdown, human (5’acctgccttcgagcagattcttcaagagagaatctgctcgaagggcactttttc3’) and mouse (5’acgggagcgaatctgcatggtcttcaagagagaccatgcagattcgctcctttttc3’) CIB1 shRNA were cloned into a mammalian FG12 expression vector that separately expresses green fluorescent protein (GFP) via a separate promoter. For control shRNA in murine ECs, human CIB1 shRNA was used, since as previously reported, it has no homology to murine CIB1 or to any sequence in the mouse genome. For control shRNA in human ECs, mouse R-Ras shRNA (5’acctctccacacagatctttcaagagagagtgtggaagagctttttc3’) was used since it has no homology to any sequence in the human genome. For CIB1 overexpression, the lentiviral vector was reconstructed to express CIB1 cDNA downstream of a cytomegalovirus (CMV) promoter. A control cDNA vector was also constructed containing only the CMV promoter. HEK293 cells
were transfected with lentivirus packaging vectors, as well as shRNA or the cDNA construct of interest. ECs were transduced with lentivirus collected from HEK293 culture supernatant at multiplicity of infection (MOI) 10 – 25. CIB1 knockdown or overexpression was confirmed via Western blotting. Densitometry with the software Quantity One (Fluor-S Multimager; Bio-Rad Laboratories, Hercules, CA) was used to determine fold change in CIB1 expression.

**Isolation of primary ECs.** Hearts and lungs of approximately 1-3 month old WT and CIB1⁻/⁻ mice were dissected from the mouse mediastinum. Organs were gently minced, collagenase-digested, and strained. The resulting cell suspension underwent positive cell sorting using PECAM-1 (BD Biosciences, San Jose CA) anti-rat IgG-conjugated magnetic beads (Invitrogen, Carlsbad, CA). Isolated cells were plated in tissue culture flasks coated with 5ng/mL fibronectin and cultured in growth media. Heart and lung ECs were then purified with a second round of positive cell sorting using ICAM-2 (BD Biosciences) coated magnetic beads. The remaining ECs were cultured for up to 4 passages.

**Boyden chamber haptotactic assay.** Haptotactic migration was performed using 8µm pore polycarbonate transwell membranes (Corning, Corning, NY) coated underneath with 25µg/mL fibronectin (Calbiochem). Cells were serum starved for at least 24h, and 2x10⁵ cells were added to the top chamber. Cells migrated in basal media (0.1% BSA and 20U heparin) supplemented with or without 250ng/mL bFGF or VEGF (R&D Systems). After 6h, non-migrating cells were removed from the topside of the membrane, and membranes were isolated, fixed, and stained with Alexa Fluor 594-conjugated phalloidin (1:1000; Invitrogen). Because the doubling time for ECs is beyond 12h, proliferation is unlikely to significantly influence this assay. To obtain
counts of migrating cells expressing pre-transduced lentiviral vectors, both Texas red and GFP fluorescence images of at least 5 random digital frames were collected using a Nikon Eclipse TE300 inverted fluorescent microscope equipped with a CoolSnap Photometrics HQ camera from Media Cybernetics with Image-Pro Plus 5.0 software. Images were transferred to NIH ImageJ software (http://rsb.info.nih.gov/ij/), where total numbers of haptotactic cells were counted and reported as mean number of cells per imaged field. For each condition, experiments were repeated at least 3 times.

**Monolayer wound healing culture assay.** EC cultures were grown to confluency in 10 cm culture plates. Cells were serum starved for at least 48 hours and wounds were created in the monolayer culture with a medium sized pipette tip. Growth media was then added and images were collected at approximately the same areas of the same wounds 0, 16, 20, and 24 hours post-wound induction. Percentage in wound area closure was measured using ImageJ software.

**Matrigel tubule formation assay.** Using a 48-well culture format, wells were coated with 150µL of GFR Matrigel (BD Biosciences). Serum starved 5 x 10^4 cells were added on top of the polymerized GFR Matrigel. Cells were then incubated in the presence of growth media, or basal media supplemented with 25ng/mL of bFGF or VEGF. Images were collected 18 hours after incubation. Oval tubule structures formed by networks of GFP-positive ECs were counted using ImageJ software. For each condition, experiments were repeated at least 3 times.

**ELISA.** For BrdU incorporation, 1x10^4 ECs were adhered in a 96-well culture format followed by serum starvation for at least 24 hours. Growth media or basal media supplemented with
25ng/mL bFGF or VEGF (R&D Systems) was then added. BrdU addition and detection at 24 hours were performed according to manufacturer’s instructions (Roche, Indianapolis, IN). For each condition, experiments were repeated at least 3 times and absorbance measured in a multi-well spectrophotometer at 450nm (Spectra MaxPlus, Molecular Devices, Sunnyvale, CA).
**Supplemental References:**


Supplemental Figure Legends:

Supplemental Figure 1
CIB1 is expressed in various mouse embryonic vascular structures and EC types. (A) Northern blot using full-length CIB1 cDNA probe confirms CIB1 mRNA expression in adult mouse lung, kidney, and testis. Additionally, we found that CIB1 mRNA is expressed in mouse ovary, spleen, and bone marrow. CIB1 mRNA expression was highest in the testis, kidney, and bone marrow. Northern blot also confirmed CIB1 mRNA expression in mouse embryonic tissue, (A and B). Specifically, we found that it is expressed at embryonic day (ED) 7.5, 8.5, and 9.5. CIB1 mRNA was highly expressed in the primitive embryonic vascular structures such as the yolk sac and placenta of ED 8.5 and 9.5 mouse embryos. In panels (A) and (B), 28S rRNA indicates loading equivalency. (C) Relative to 1ng of CIB1 (first lane), Western blot revealed that CIB1 is expressed in various types of ECs including transformed (mouse embryonic heart endothelial cells; MECs and bovine aortic endothelial cells; BAECs), primary (human aortic endothelial cells; HAECs, HUVECs, human coronary artery endothelial cells; HCAECs, and human pulmonary artery endothelial cells; HPAECs), embryonic (MECs), macrovascular (BAECs and HAECs), and microvascular ECs (HUVECs, HCAECs, and HPAECs).

Supplementary Figure 2
Lentiviral gene delivery for CIB1 knockdown and overexpression. (A) For knockdown, lentivirus was packaged with a double-cassette FG12 vector expressing either control shRNA or CIB1 shRNA, and green fluorescent protein (GFP) via a separate promoter (vectors 1 & 2). For CIB1 overexpression and overexpression control, similar double-cassette vectors were used with
or without CIB1 cDNA (vectors 3 & 4). (B) Stable knockdown and overexpression of CIB1 in MECs was confirmed via Western blot. (C) Similarly, relative to 5ng of CIB1 (lane 1), HUVECs transduced with vectors 1 demonstrated stable knockdown compared to cells transduced with vector 2 and HUVECs that were untransduced (UT). Densitometry analysis of (B) and (C) confirmed that CIB1 knockdown was up to 80-90%, respectively, and CIB1 overexpression in MECs was up to 600%.

**Supplemental Figure 3**

Efficient lentiviral transduction of MECs and HUVECs. (A) Differential interference contrast (DIC) and GFP fluorescent images of confluent monolayers of MECs that were transduced with lentivirus packaging one of four different vectors (control shRNA, CIB1 shRNA, control cDNA, or CIB1 cDNA vectors). Three days after infection, GFP expression was detected in approximately 95% of cells (95% transduction efficiency). (B) Similarly, DIC and GFP fluorescent images of HUVECs that were transduced with lentivirus packaging either control shRNA or CIB1 shRNA vectors. At high and low magnifications, transduction efficiency was approximately 100%. Untransduced MECs and HUVECs do not express GFP, (A and B). Short scale bars; 40µm, and long scale bars; 50µm.

**Supplemental Figure 4**

Loss of CIB1 decreases EC monolayer wound healing. (A) DIC (left) and GFP (right) fluorescent images of monolayer wounds created in confluent cultures of MECs transduced with control shRNA or CIB1 shRNA. GFP images confirm MEC transduction. Dotted lines outline the wound boundaries. Although monolayer wounds in control MECs completely resolve by 20
hours post-wound induction, CIB1 knockdown MEC monolayer wounds only resolve by 48%. Scale bar, 100µm. (B) Accordingly, monolayer wounds in WT MHECs completely resolved by 24 hours, whereas CIB1 MHECs only resolve by 64%. Scale bar, 40µm.

**Supplemental Figure 5**

Retinal vascular development is normal in CIB1−/− retinas. (A) WT (top images) and CIB1−/− (bottom images) P2 retinas. Interconnected hyaloid vasculature can be seen superimposed upon avascular retinas. Emerging retinal vasculature can be seen at the center of the retina at the optic cup (outlined in blue). (B) P4 retinas still demonstrate superimposed hyaloid vasculature and both WT and CIB1−/− retinas show expanding retinal vasculature. (C) Hyaloid vasculature has regressed in P8 WT and CIB1−/− retina and they are both almost fully vascularized. (D) Vessels reach the perimeter of WT and CIB1−/− P12 retinas, and normal vascular pruning around established retinal arterioles (E). (F) Adult, 6 week old WT and CIB1-KO retinas are fully vascularized and confocal microscopy shows the formation of all three retinal layers; superficial, indicated by large arrowhead; intermediate, indicated by small arrowhead; and deep, indicated by (*)
Supplemental Figure 2:

A

1: LTR hU6 CIB1 shRNA UbiC GFP LTR
2: LTR hU6 Control shRNA UbiC GFP LTR
3: LTR CMV CIB1 UbiC GFP LTR
4: LTR CMV UbiC GFP LTR

B

kDa 1 2 WB: CIB1 ERK1/2 GAPDH
20- 37- 20- 37-

C

kDa CIB1 UT WB: CIB1 α-tubulin
20- 50-
Supplemental Figure 3:

A

Untransduced | Control shRNA | CIB1 shRNA | Control cDNA | CIB1 cDNA

DIC

GFP

MEC

B

Untransduced | CIB1 shRNA | Control shRNA

DIC

GFP

HUVEC
Supplemental Figure 4: