Human Tribbles-1 Controls Proliferation and Chemotaxis of Smooth Muscle Cells via MAPK Signaling Pathways*

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Migration and proliferation of smooth muscle cells are key to a number of physiological and pathological processes, including wound healing and the narrowing of the vessel wall. Previous work has shown links between inflammatory stimuli and vascular smooth muscle cell proliferation and migration through mitogen-activated protein kinase (MAPK) activation, although the molecular mechanisms of this process are poorly understood. Here we report that tribbles-1, a recently described modulator of MAPK activation, controls vascular smooth muscle cell proliferation and chemotaxis via the Jun kinase pathway. Our findings demonstrate that this regulation takes place via direct interactions between tribbles-1 and MKK4/SEK1, a Jun activator kinase. The activity of this kinase is dependent on tribbles-1 levels, whereas the activation and the expression of MKK4/SEK1 are not. In addition, tribbles-1 expression is elevated in human atherosclerotic arteries when compared with non-atherosclerotic controls, suggesting that this protein may play a role in disease in vivo. In summary, the data presented here suggest an important regulatory role for trb-1 in vascular smooth muscle cell biology.

Injury to the arterial wall initiates a series of changes in specialized molecular and cellular events that not only contribute to wound healing but also to the pathogenesis of atherosclerosis, the presentation of acute coronary syndromes, and the complications of percutaneous coronary intervention. An important initiating event appears to be endothelial cell dysfunction or cell death caused by local abnormalities. These events result in the production and release of a number of inflammatory cytokines and chemokines. In pathogenic conditions, elevated levels of inflammatory mediators can lead to migration and proliferation of vascular smooth muscle cells (VSMC) to form a neointima. These cellular responses are mediated via the coordinated action of various second messenger pathways, including activation of mitogen-activated protein kinase (MAPK) cascades and IκB kinases. Activation of these systems has been reported in several pathological conditions of vessel walls (1–3). MAPKs are involved in the regulation of development, cell activation, proliferation, and vascular contraction (4–7). Further, they are central in regulating VSMC activation. It has been demonstrated that activation of MAPK cascades occurs in response to a wide range of stimuli, including pro-inflammatory cytokines, growth factors, mechanical stimuli (stress) (8), and integrin-dependent cell/matrix interactions (9, 10). MAPKs are classified into at least three distinct groups (JNK, p38, and extracellular signal-regulated protein kinases (ERK) kinases) and can be activated via a variety of upstream kinases, MAPKKs. In VSMC, Jun kinases (JNK) and p38 MAPKs have been implicated in responses primarily to stress (heat, hypoxia, chemical, oxidative, etc.) and pro-inflammatory cytokines, whereas ERK kinases primarily respond to mitogenic stimuli such as growth factors (PDGF) (11), oxidized low density lipoprotein (12), or angiotensin II (reviewed in Ref. 13). However, in most cases, a given stimulus will activate more than one group of MAPKs. The specific contribution of each MAPK pathway to a physiological response varies from cell type to cell type. In some cases, MAPK pathways can cooperate, but they can antagonize in others (14, 15). However, the mechanisms that are responsible for these differences in MAPK responses in VSMC are largely unknown.

We have recently reported the identification (16, 17) and characterization of a novel protein family, human tribbles (trib), as regulators of MAPKK activity (16, 18). We have shown that trib-1 and trib-3 are able to bind to various MAPKKs and that their concentration regulates preferential activation of the different MAPK pathways, presumably leading to different cellular responses (18). Drosophila and Xenopus Tribbles have been shown to regulate cell cycle progression during embryonic development (19–22). Murine trib-3 has been demonstrated to

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3 The abbreviations used are: VSMC, vascular smooth muscle cells; MAPK, mitogen-activated protein kinase; pMAPK, phosphorylated MAPK; MAPKK, MAPK kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; PDGF, platelet-derived growth factor; siRNA, small interfering RNA; siTrb-1, small interfering Trb-1; YFP, yellow fluorescent protein; EFYP, enhanced YFP; PCA, protein fragment complementation assay; LPS, lipopolysaccharide; HD, ischemic heart disease; DCM, dilated cardiomyopathy; IL, interleukin; IL-RA, IL receptor antagonist; qRT-PCR, quantitative reverse transcription-PCR; TNFα, tumor necrosis factor α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; hASMC, human aortic smooth muscle cells.
inhibit insulin-dependent activation of Akt and was suggested to play a role in the development of diabetes in a mouse model (23, 24). However, recent conflicting data suggest that this proposed role may require further clarification (25). Further, the importance of human tribbles proteins in cell physiology has not been evaluated. Here we report that trb-1 is selectively overexpressed in chronically inflamed human atherosclerotic arteries and that it regulates vascular smooth muscle cell proliferation and chemotaxis via the JNK pathway. These observations define trb-1 as a novel, central regulator of VSMC function.

**EXPERIMENTAL PROCEDURES**

All the experiments described in this study were performed multiple times (n ≥ 2), and representative data sets are shown. 

**Ethics**—The human samples were obtained under the ethical approval granted by the North Sheffield Research Ethics Committee. This study conforms to the principles outlined in the Declaration of Helsinki.

**Plasmids and siRNA**—Trb-1 overexpression plasmid has been described before (18). siRNA SmartPool against human trb-1 was purchased from Dharmacon and used according to the manufacturer’s recommendation. Plasmids for yellow fluorescent protein (YFP)-based protein fragment complementation assay (PCA) (42, 43) were a kind gift of Prof. S. Michnik and have been described before (26).

**Cell Culture and the Stimulation with LPS**—VSMC were purchased from Cascade Biologics and maintained in Medium 231 with smooth muscle growth supplement (all from Cascade Biologics). VSMC were plated in 6-well plates at an initial density of 0.5 × 10^5 cells/well in 2 ml of complete growth medium. 24 h after transfection, cells were stimulated by 100 ng/ml LPS, as stated in the legend for Fig. 4. HeLa cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum (BioWhittaker), 5 mM g/ml penicillin and streptomycin (Sigma).

**Artery Biopsies**—Coronary arteries from patients with a diagnosis of ischemic heart disease (IHD, n = 8) and dilated cardiomyopathy (DCM, n = 6) were harvested from transplantation recipients at the time of surgery. Patients diagnosed as having DCM were free of chronic arterial disease before transplantation, as assessed by coronary angiography and histological analysis by a cardiac pathologist (S. Kim Suvarna) using the American Heart Association (AHA) classification system (27–29). All patients were men and white in origin. Mean ages were 55.75 ± 5.11 for IHD and 48.5 ± 10.23 for the DCM group. Mean AHA histological grades of disease were 6.42 for the IHD and 3.16 for the DCM group, respectively.

**RNA Isolation and Quantitative Real-time PCR Analysis**—Total RNA was extracted from human artery biopsies and VSMC using RNaseasy kit (Qiagen) according to the manufacturer’s protocol. 2 μg of RNA was reverse-transcribed into first strand cDNA and used immediately for qRT-PCR. Gene expression was analyzed by qRT-PCR using ABI prism 7900 (Applied Biosystems). Probes for human trbs, IL-1β, IL-RA, TNFα, and GAPDH were synthesized by Sigma Genosys with FAM at the 5’-end and TAMRA at the 3’-end. The sequences of all primers and probes used are listed in Table 1.

To quantify transcripts for genes of interest, we used the GAPDH transcript as an internal control, and each sample was normalized with respect to GAPDH transcript content. Standard curves for the nine genes and GAPDH mRNAs were generated using serially diluted solution of plasmids incorporating each gene as a PCR template. All assays were performed in duplicate, and the average values were used for analysis.

**Transfections**—Transfections were performed using Nucleofector (Amaza) using program U-25 and Nucleofector solution for VSMC (Amaza). For most experiments, 1.0 × 10^6 cells were used per nucleasection. The rate of transfection was >40 as judged by enhanced green fluorescent protein expression, measured by fluorescent microscopy (data not shown). Polyfect (Qiagen) was used for transfection of HeLa cells according to the manufacturer’s instructions.

**Proliferation Assay**—Transfected VSMC were placed on 96-well culture plates and further cultured for 24 h. Cells were then treated with [3H]thymidine (1 μCi/well) and/or MAPK inhibitors for 6, 24, and 30 h. ERK MAPK inhibitor (PD98059), p38 MAPK inhibitor (SB203580), and JNK MAPK inhibitor (SP600125) were purchased from Calbiochem and used at 20 μM for ERK and JNK MAPK inhibitors and 0.2 μM for p38 MAPK inhibitor. After treatment, [3H]thymidine incorporation was measured by liquid scintillation counting.

**In Vitro Wound-healing Assay and Time-lapse Video-Microscopy**—Cells were transfected as described above, seeded into 35-mm culture dishes, and incubated overnight. Confluent cell monolayers were wounded by removing a strip of cells from the plate surface with a standard 1-ml pipette tip. Wounded monolayers were washed with phosphate-buffered saline to remove non-adherent cells and replaced with fresh complete growth medium. Time-lapse video-microscopy (30, 31) was used to film migration at the wound edges for 24 h (1 frame every 2.5 min).

The number of cells crossing a line marking the wound edges was counted over the 24-h period. (see Fig. 2D). 15 randomly picked cells from the wound edges were tracked over a 1-h period. The on-screen distance traveled was measured to cal-

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**TABLE 1**

| Primers used in this study | Target | Primer type | Sequence (5’–3’) |
|----------------------------|--------|-------------|-----------------|
| htrb-1                     | Forward | CCCAAAAGACAGGCCTCTT | CTTGGAGACGGGTA-TAMRA |
| htrb-2                     | Forward | CATAACAGGTCTACCCCC | Reverse |
| htrb-3                     | Forward | GGCGATCGTGTCCTGGC | Reverse |
| IL-1β                      | Forward | ACAGATGGATCTCCCTCCA | Reverse |
| IL-RA                      | Forward | GAAAGTGGCCCTGGTCCG | Reverse |
| GAPDH                      | Forward | GCCATCCGCTGGCCACT | Reverse |
|                            | Probe   | FAM-CTCCCTCCACCCACT | TAMRA |
|                            | Probe   | FAM-CCTGCTCCACCCACT | TAMRA |
|                            | Probe   | FAM-CCTGCTCCACCCACT | TAMRA |
culate migration speed (mm/h) irrespective of direction. (see Fig. 2E)

Western Blotting—Anti-phospho-MAPK and anti-MAPK antibodies were purchased from Cell Signaling Technology and were used according to the manufacturer’s recommendations. Anti-β-actin antibody was from Dako. Between probing for the various proteins, membranes were stripped by Re-Blot Plus mild solution (Chemicon). Membranes were developed with SuperSignal West Pico chemiluminescent substrate, and signals were quantified by Chemigenius gel documentation system (Syngene). pMAPK and MAPK signals were expressed as a ratio to β-actin levels within the same sample.

Immunostaining—Serial 4-μm thick paraffin-embedded human artery sections were stained for Trb-1 using standard techniques. Trb-1 primary antibody (Millipore-Upstate) was applied overnight at 4 °C. Sections were then exposed to goat biotinylated anti-rabbit secondary antibody (Vector laboratories) for 30 min at room temperature. Vectastain ABC reagent (Vector Laboratories) was added and incubated at room temperature for 30 min. Slides were then immersed in 0.25 mg/ml 3,3′-diaminobenzidine, activated with hydrogen peroxide, and counterstained with Carazzi’s hematoxylin. Primary antibody was omitted from the negative controls.

RESULTS

Tribbles Expression Is Regulated in Response to an Inflammatory Stimulus in hASMC—Smooth Muscle Cell proliferation is a key event in the healing response to injury, a process that is initiated by inflammatory stimuli (32). To identify whether tribbles expression is regulated in vascular cells under inflammatory conditions, we stimulated human aortic smooth muscle cells (hASMC, Fig. 1A) and human umbilical vein endothelial cells (not shown) with LPS and measured tribbles mRNA expression levels by using qRT-PCR. IL-1β expression was measured as positive controls in the same samples. We found that trb-1 was selectively and transiently up-regulated by LPS treatment in hASMC (Fig. 1A) but not in human umbilical vein endothelial cells (not shown). Previous reports (33) suggest that tribbles proteins may be unstable and expressed at low levels; therefore, mRNA levels are likely to correlate well with protein expression. We have, therefore, investigated the biological relevance of altered trb-1 levels on hASMC under inflammatory conditions.

To assess the role of trb-1 in LPS-stimulated hASMC, we transiently transfected these cells with a trb-1 expression plasmid or a pool of anti-trb-1 siRNA oligonucleotides and measured proliferation and migration in response to PDGF. However, first we evaluated the potential impact of culture conditions on trb-1 expression (Fig. 1C) and the specificity of the siRNA pool (Fig. 1D) used for targeting trb-1 mRNA. We have found that although trb-1 siRNA had the desired activity both under standard culture conditions (10% fetal calf serum) and when cells were serum-starved after transfection (0.5%), the expression levels of this gene and, indeed, the other tribbles as well (not shown) were significantly affected by the serum concentration. This is in line with previous work, demonstrating that expression of members of mammalian trb family is modulated through metabolic signals (34, 35). Therefore, we have used 10% fetal calf serum in all our experiments to minimize inter-experimental variations. In addition, the siRNA pools showed high specificity toward trb-1 and did not alter significantly trb-2 and -3 expression (Fig. 1D). In line with these results, siTrb-1 treatment resulted in a substantial decrease in trb-1 protein levels (Fig. 1E).

Tribbles-1 Regulate Specific hASMC Cellular Functions—To investigate the role of trb-1 in hASMC function, we raised or suppressed trb-1 levels by transient transfection of hASMC cells with trb-1 expression plasmid or siRNA and measured proliferation by [3H]thymidine incorporation (Fig. 2, A and B). We observed a modest anti-proliferative effect at 48 h after transfection (24-h time point in the proliferation assay) when
Trb-1 was overexpressed. In contrast, depletion of trb-1 results in a significant increase in proliferation rate at the same time point (Fig. 2B). However, this assay measures the net effect of cell death and proliferation. To confirm that the observed increase in \[^{3}H\]thyridine incorporation is due to an increased mitosis rate, we performed time-lapse video imaging on control and si-trb-1-treated cells and calculated the rate of mitosis (Fig. 2C). The data obtained by both methods were in agreement. In addition, there was no difference in the observed rate of apoptosis between treatment, further supporting a specific tribbles effect on proliferation. Using the same time-lapse assay, we also measured VSMC migration in a wound-healing assay (Fig. 2D, E). These data show that neither the number (Fig. 2D) nor the migration speed (Fig. 2E) of cells migrating into the wound was affected upon depletion of trb-1.

As trb-1 acts via modulating MAPK activation, and this signaling system is known to govern VSMC proliferation, we used selective MAPK inhibitors to block individual pathways and investigated their involvement in the trb-1-regulated proliferation (Fig. 2F). These data demonstrate that blocking of JNK but not p38 pathways suppresses VSMC proliferation. In line with previous reports, inhibiting ERK activation also caused a significant inhibition of proliferation (36).

To further investigate the impact of trb-1 levels in hASMC function, chemotaxis migration of these cells was measured in a transwell migration assay, in response to PDGF (Fig. 2G). Depletion of trb-1 led to an increase in transmigrated cells. Further, inhibition of the JNK pathway abrogated the effect of si-trb-1 treatment, suggesting that trb-1 may be a negative regulator of hASMC chemotaxis via inhibitory activity of the JNK pathway.

Activation of vascular smooth muscle cells by inflammatory signals leads to the production of a number of cytokines, including TNFα and transforming growth factor β (TGFβ) protein levels (I) was evaluated by qRT-PCR and enzyme-linked immunosorbent assay, respectively.
ference in the dynamics or the amplitude of cytokine expression at the TNFα mRNA (Fig. 2H) and transforming growth factor β protein levels (Fig. 2I), implying that trb-1 may be a specific regulator of VSMC proliferation and migration.

**Overexpressed Trb-1 Blocks AP-1 Activation and Trb-1 Depletion Leads to Constitutive JNK Activation**—To gain mechanistic insight into the regulation of VSMC proliferation via the JNK/AP-1 pathways and trb-1, VSMC was transiently transfected with an AP-1 reporter plasmid, activated by overexpressed MEKK1 in the presence and absence of overexpressed trb-1 (Fig. 3A). The results demonstrate that activation of AP-1 can be blocked by overexpressed trb-1 in VSMC, in line with our previous report in HeLa cells (18).

Next, the activation of the various MAPKs was assessed in control and trb-1-depleted cells. Phosphorylated MAPK (pMAPK, the activated form) and total MAPK levels were investigated in response to LPS treatment by Western blotting. pMAPK (Fig. 3B) and total MAPK (Fig. 3C) levels were normalized to β-actin and expressed as relative units. In agreement with our previous report (18), alterations in trb-1 levels had a differential impact on the various MAPK pathways. However, these effects were different in VSMCs when compared with those observed in HeLa cells (18). In VSMC, the amount of phospho-p38 protein but not the dynamics of activation was influenced by altered trb-1 expression. In contrast, whereas ERK and JNK pathways were also sensitive to altered trb-1 expression, these were no longer up-regulated by LPS stimulation. Indeed, modulation of trb-1 expression caused phosphorylation of both MAPKs to decrease once stimulated. In addition, depletion of trb-1 mRNA by the siRNA constructs led to an increase in phosphorylation of JNK in the absence of any stimulus (Fig. 3B, middle graph, zero time point), suggesting that normal trb-1 levels inhibit the activation of this pathway in the non-stimulated state. However, no significant alterations
in total MAPK levels were observed in cells, where trb-1 levels were perturbed (Fig. 3C). These observations are compatible with the hypothesis that MAPK scaffold levels are key determinants of pathway activation (42) and that tribbles may have a scaffold-like function as proposed previously (18). According to these models, both up-regulation and down-regulation of scaffold levels may lead to impaired activation of signaling systems. We have seen such effects on the JNK and ERK pathways in VSMC, similarly to our previous studies on HeLa cells (18).

**Trb-1/MKK4 Interaction Is Key to the Regulation of hASMC Proliferation**—We have previously shown in biochemical assays that tribbles proteins interact with MAPKKs and regulate their activity (18). However, since many aspects of tribbles action appear to be cell type-specific (for recent reviews, see Refs. 43 and 44), we set out to investigate details of trb/MAPKK interactions in hASMC. Our data point to the JNK pathway as a key system in regulating proliferation and chemotaxis in these cells, in a trb-1-dependent manner. Therefore, we characterized the involvement of MKK4/SEK-1 and MKK7, the two known MAPKKs, which lead to activation of JNK. Our results show that MKK4 but not MKK7 is expressed in these cells and that this expression pattern is not influenced by sitrb-1 treatment (Fig. 4A). We also found that the phosphorylation of MKK4 was not affected by depletion of trb-1 (Fig. 4B), suggesting that trb-1 directly interferes with MKK4 activity, rather than with activation and/or expression. To confirm the direct role of MKK4 in hASMC proliferation, we performed a [3H]thymidine incorporation assay in siMKK4-treated cells (Fig. 4C), as before. In line with our above model, the data demonstrate a positive role for MKK4 since depletion of this protein led to a decrease in proliferation rate.

**FIGURE 4.** MKK4-trb-1 interaction controls hASMC proliferation. A, expression of MKK4 and MKK7 in hASMC and the impact of trb-1 knockdown on the protein levels of these MAPKKs were assessed by Western blotting. B, activation of MKK4 (30 min, 100 ng/ml LPS) was evaluated under normal and reduced trb-1 levels. pMKK4 values were normalized to actin and expressed as a ratio. C, the ability of MKK4 to control hASMC proliferation rate was measured as on Fig. 2 (24 h after transfection). The efficiency of MKK4 knockdown was verified by Western blot (upper panel), si contr., control. D, physical interaction between MKK4 and trb-1 in hASMC was investigated by PCA. As positive controls, enhanced green fluorescent protein (EGFP) expression plasmid (left upper panel) and “zipper-PCA” (left lower panel, zip-V1 and zip-V2) constructs were used. MKK4 was fused to the N-terminal fragment of Venus-YFP (V1), whereas trb-1 was expressed in fusion with the C-terminal fragment of Venus-YFP (V2). Representative cells show interaction between MKK4 and trb-1 (right panels). E, to further confirm association between trb-1 and MKK4, co-immunoprecipitation was performed using trb-1-Myc expression construct. Lane 1, detection of MKK4 in a whole cell lysate. Lane 2, detection of MKK4 after anti-Myc pull-down. F, the impact of the N- and C-terminal domains of trb-1 on interaction with MKK4 in live cells and the location of the trb-1/MKK4 complex was assessed by PCA. G, the structure of trb-1 mutants and the positions of the N-terminal (N-term.) and C-terminal (C-term.) deletions is shown. aa, amino acids. H, to confirm the specificity of MKK4/trb-1 interaction, a fluorescence-activated cell sorter was used to demonstrate the specific interaction between trb-1 and MKK4 in HeLa cells. Similarly to Fig. 5E, an increasing dose of non-fluorescent trb-1 expression plasmid was co-transfected to compete out the labeled protein from the fluorescent complex. Further, no interaction was detected between control plasmids and either MKK4-V1 or trb-1-V2. I, as a further control, an increasing dose of trb-1 expression plasmid (unlabeled) was co-transfected in HeLa cells with the above two constructs (left), and the average total fluorescence per cell was measured (right) by fluorescent microscopy.
live hASMC, we used a YFP-based PCA (45, 46) and co-immunoprecipitation. The Venus variant of YFP was used in this assay since it provides a higher signal than EYFP. MKK4 and Trb-1 were fused to the N-terminal fragment of Venus YFP (V1) or to the C-terminal portion of Venus YFP (V2), respectively. The two expression constructs were co-transfected, and the YFP signal was visualized by fluorescent microscopy (Fig. 4D). These data demonstrate that the MKK4/trb-1 complex is located predominantly in the nucleus of hASMC. In line with this finding, a co-immunoprecipitation experiment further confirmed the interaction between MKK4 and trb-1 (Fig. 4E).

We have shown recently that the N-terminal region of trb-1 governs the intracellular localization of the protein (17). We wondered, therefore, whether the same domain influenced the location of the MKK4/trb-1 complex. Truncated trb-1 forms, lacking the N-terminal, the C-terminal, or both domains (Fig. 4F) were expressed as V2 fusion proteins (Fig. 4E). The results show that the nuclear localization of the complex is critically dependent on the presence of the N-terminal trb-1 domain. Mutants lacking this domain still showed an interaction with MKK4, but the signal was no longer preferentially nuclear. Further, these experiments demonstrate that the central, kinase-like domain of trb-1 is sufficient for interaction with MKK4. This is in line with our previous report, where we showed that expression of this domain was sufficient to inhibit of AP-1 activation via trb-1 (17).

To confirm specificity of the observed interaction, several control experiments were performed. Co-expression of an increasing amount of “unlabeled” trb-1 led to a dose-dependent elimination of the YFP signal as detected by fluorescence-activated cell sorter (Fig. 4G) or by fluorescent microscopy (Fig. 4H). In addition, neither MKK4-Venus nor trb-1-Venus fusion proteins interacted with their zip-Venus counterparts (these were used as positive control constructs in the system) (Fig. 4G), which further supports the specific nature of this interaction.

**Tribbles-1 Expression in Atherosclerotic Arteries**—Proliferation of vascular smooth muscle cells is one of the hallmarks of the development of chronic diseases of the vessel wall. To evaluate the potential role of tribbles in human disease, we studied segments of whole artery wall taken from the explanted hearts of patients undergoing cardiac transplantation for IHD and characterized tribbles expression. Coronary arteries from patients with non-ischemic DCM were used as controls. To
quantify potential differences in trb expression levels between the two groups, mRNA levels of known pro- and anti-inflammatory cytokines (IL-1β and IL-1ra) and tribbles 1–3 were quantified by using qRT-PCR (Fig. 5). As in our previous work (47), we detected a "pro-inflammatory phenotype" in the atherosclerotic group. Expression of trb-1 but not of trb-2 was significantly raised in the IHD group (Fig. 5A), whereas trb-3 expression was not detected in these samples (not shown).

To visualize trb-1 expression in vivo, sections of a human coronary artery were stained by anti-trb1 antibody (Fig. 5B). The majority of trb-1-specific staining was detected in the intimal and medial areas. Of note, nuclear and/or cytoplasmic trb-1 staining was observed in medial VSMC.

DISCUSSION

In this study, we have evaluated the importance of tribbles proteins, particularly trb-1, in the cellular responses of vascular smooth muscle cells to inflammatory stimuli. Taken together, our experiments demonstrate that trb-1 is found in VSMC in vivo and that expression levels are key in modulating the extent of VSMC proliferation and chemotaxis. In contrast, VSMC migration speed in a wound-healing assay and the production of cytokines are not affected by altered trb-1 levels, suggesting a specific physiological role for this protein. Although a body of literature supports the involvement of ERK and JNK MAPK pathways in VSMC chemotaxis, the role of these second messenger systems in wound-healing assays (in the absence of chemokines) is much less understood. Our data imply that the two processes might be differentially regulated. However, further detailed studies will be needed to clarify this.

Blockade of specific MAPK pathways by pharmacological inhibitors pointed to the JNK pathway as a major regulator of VSMC proliferation. In line with the model where trb-1 controls JNK activation (Fig. 6), we found that si-trb-1 treatment of VSMC led to the spontaneous activation of JNK (Fig. 3B). Therefore, we have investigated the detail of the interaction between JNK-activating kinases and trb-1. Of the MAPK kinases, which are known to activate JNK, MKK4 but not MKK7 was found to be expressed in VSMC. Using a recently developed technique (PCA), we were able to visualize the interaction between trb-1 and MKK4 in live VSMC cells. To our knowledge, this is the first use of this powerful technique in studies of primary vascular cells. A number of controls demonstrate the specificity of the observed interaction. Further, we were able to show that the intracellular localization of the trb-1/MKK4 complex is dependent on the N-terminal domain of trb-1. This is in line with our previous findings where we demonstrated that the N-terminal domain of trb-1 is essential for
nuclear localization. However, clarification of the functional relevance of this observation requires further studies. The use of truncated trb-1 proteins in PCA demonstrates that the kinase-like domain of trb-1 is essential for its ability to interact with MAPKKs. Since this domain is similar to that of the MAPKs (MAPKK substrates), a plausible hypothesis for the molecular mechanism of trb action is that tribbles may compete for the binding site with the MAPKs, thus regulating their activation. This model may explain why evolution preserved a catalytically inactive kinase domain from unicellular organisms to mammals (43, 44).

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