Ges, A Human GTPase of the Rad/Gem/Kir Family, Promotes Endothelial Cell Sprouting and Cytoskeleton Reorganization

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Abstract. Rad, Gem/Kir, and mRem (RGK) represent a unique GTPase family with largely unknown functions (Reynet, C., and C.R. Kahn. 1993. *Science.* 262: 1441–1444; Cohen, L., R. Mohr, Y. Chen, M. Huang, R. Kato, D. Dorin, F. Tamanoi, A. Goga, D. Afar, N. Rosenberg, and O. Witte. *Proc. Natl. Acad. Sci. USA*. 1994. 91:12448–12452; Maguire, J., T. Santoro, P. Jensen, U. Siebenlist, J. Yewdell, and K. Kelly. 1994. *Science.* 265:241–244; Finlin, B.S., and D.A. Andres. 1997. *J. Biol. Chem*. 272:21982–21988). We report that Ges (GTPase regulating endothelial cell sprouting), a human RGK protein expressed in the endothelium, functions as a potent morphogenic switch in endothelial cells (ECs). Ges function is sufficient to substitute for angiogenic growth factor/extracellular matrix (ECM) signals in promoting EC sprouting, since overexpression of Ges in ECs cultured on glass leads to the development of long cytoplasmic extensions and reorganization of the actin cytoskeleton. Ges function is also necessary for Matrigel-induced EC sprouting, since this event is blocked by its dominant negative mutant, Ges^{T94N}, predicted to prevent the activation of endogenous Ges through sequestration of its guanine nucleotide exchange factor. Thus, Ges appears to be a key transducer linking extracellular signals to cytoskeleton/morphology changes in ECs.

Key words: angiogenesis • actin • morphology change • Matrigel

Introduction

Rad, Gem/Kir, and mRem (RGK)[1] are members of a newly emerged Ras-like GTPase family with many unique characteristics, including the following. Tissue-specific expression: Rad and mRem are expressed most abundantly in skeletal muscle, heart, and lung, but rarely in brain, liver, and pancreas (Reynet and Kahn, 1993; Finlin and Andres, 1997), whereas Gem/Kir expression is high in thymus, spleen, and kidney, but low in skeletal muscle and heart (Maguire et al., 1994). Transcriptional regulation in disease or models of disease pathogenesis: Rad is overexpressed in the skeletal muscle of type II diabetic humans (Reynet and Kahn, 1993); Gem is transiently induced in peripheral blood T cells (Maguire et al., 1994) and endothelial cells (ECs; Vanhove et al., 1997) by PMA/phytohemagglutinin and proinflammatory cytokines, respectively; Kir is upregulated in pre-B cells transformed by abl tyrosine kinase oncogene (Cohen et al., 1994); and mRem is transiently repressed in tissues of mice injected with lipopolysaccharide (Finlin and Andres, 1997). Unique GTP-binding domains (G domain): the RGK proteins contain only two (G3 and G4) of the four conserved G domains and lack all of the residues critical for GTP hydrolysis in other GTPases (Bourne et al., 1991). The conserved Gly in the G1 domain (G^{12} in Ras) is replaced by Pro or Gln, and the entire conserved G2 domain ($DTAGQ^{61}$ in Ras) is replaced by a sequence motif DXWE. These alterations in the RGKs' G domains are consistent with their low intrinsic GTPase activity (Cohen et al., 1994; Zhu et al., 1995) and suggest a distinct mechanism for the GTPase activating protein-catalyzed GTP hydrolysis (Zhu et al., 1995). A conserved calmodulin-binding domain: the RGKs interact with calmodulin in a Ca^{2+}-dependent manner, suggesting their involvement in Ca^{2+} signaling (Fischer et al., 1996; Moyers et al., 1997). A conserved

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[1]*Abbreviations used in this paper:* EC, endothelial cell; ECM, extracellular matrix; EST, expressed sequence tag; G domain, GTP-binding domain; GEF, guanine nucleotide exchange factor; Ges, GTPase regulating endothelial cell sprouting; GFP, green fluorescence protein; HPRT, hypoxanthine guanine phosphoribosyl transferase; HUAEC, human umbilical cord artery endothelial cell; ISH, in situ hybridization; RGK, Rad, Gem/Kir, and mRem; RT, reverse transcription.

COOH-terminal domain (KSKCHN/DLA/SVL): initially speculated to be a novel isoprenylation motif (Reynet and Kahn, 1993; Maguire et al., 1994), but later shown not to be modified by isoprenylation (Del Villar et al., 1996; Bilan et al., 1998).

Despite their highly conserved structural and biochemical properties, functional evidence to suggest a unified mechanism of action for the RGK proteins has been limited. It was reported that Rad overexpression inhibited glucose uptake in muscle and fat cells (Moyers et al., 1996), that Gem overexpression significantly reduced the number of selectable colonies in NIH 3T3 (Maguire et al., 1994; Vanhove et al., 1997), and that Kir overexpression induced invasive pseudohyphal growth in *Saccharomyces cerevisiae* (Dorin et al., 1995). However, the underlying biological functions for this family of GTPases are still largely unknown.

In this paper, we report the identification and characterization of Ges (GTPase regulating endothelial cell sprouting), a human RGK protein expressed in the endothelium. We show that Ges function in ECs is both sufficient and necessary to promote EC sprouting, a phenotype that mimics EC morphology change during angiogenesis in vivo and is induced in vitro only by combined signals from extracellular matrix (ECM) and angiogenic growth factors. Our findings indicate that Ges is a key transducer linking extracellular signals to downstream events, including EC sprouting, one of the hallmarks of angiogenesis.

Figure 1. Ges sequence and alignment with other RGK members. a, Full-length nucleotide sequence of Ges. Lower case represents noncoding region, upper case represents coding region. The two polyadenylation signals (aataaa) are underlined. Asterisks (*) indicate the sites where poly-A tails were added. b, Predicted amino acid sequence of Ges and comparisons with mRem, Gem, and Rad. Identical sequence is shaded. Dots indicate gaps inserted to allow for optimal sequence alignment. The conserved GTP-binding domains (G1–G4), calmodulin-binding domain, and COOH-terminal motif are boxed. The four domains with divergent sequence, N-hypervariable domain, Switch I, Switch II, and C-variable domain, are underlined. Ges sequence is available from GenBank/EMBL/DDBJ under accession number AF152863.

Materials and Methods

Database Search and Library Screening

Sequence similarity search of a proprietary expressed sequence tag (EST) database (LIFESEQ; Incyte Pharmaceuticals) was conducted using the conserved COOH-terminal motif in the RGK family (KSKSCHN/DLA/SVL). One EST corresponding to nucleotides 1,054–1,510 in the Ges sequence (Fig. 1 a) was identified in a cDNA library from the knee synovial membrane tissue of an 82-yr-old female with osteoarthritis. A ^{32}P-labeled probe corresponding to nucleotides 1,064–1,277 was generated by PCR for library screening. Approximately 600,000 recombinant plaques from a human lymph node λgt10 cDNA library (CLONTECH Laboratories, Inc.) were screened according to manufacturer's protocol, and three positive clones were obtained.

Northern Blot Analysis

Probes for Northern blot analysis were PCR products corresponding to nucleotides 1–523 or 1,064-1,511 in the Ges sequence chosen for their lack of homology with other RGK members (Fig. 1 a), both of which produced identical results. Human multiple tissue blots (2 μg mRNA/lane; CLONTECH Laboratories, Inc.) were prehybridized in ExpressHyb (CLONTECH Laboratories, Inc.) at 68°C for 30 min, hybridized at 68°C for 2 h with ^{32}P-labeled Ges probe (specific activity $\sim 6.0 \times 10^8$ cpm/μg), washed (20 min/wash) twice at room temperature with 2× SSC, 0.05% SDS, twice at 50°C with 0.1× SSC, 0.1% SDS, and exposed on X-ray film at −70°C for 1 wk. The blot was then stripped by boiling in 0.5% SDS for 10 min, and hybridized with a β-actin probe (CLONTECH Laboratories, Inc.) following the same procedure.

In Situ Hybridization (ISH)

These studies were carried out under contract with LifeSpan BioScience Inc. Ges antisense and sense control riboprobes corresponding to nucleotide 1,064–1,277 in the Ges sequence (Fig. 1 a) were generated and labeled with digoxigenin. Tissue sections from paraffin blocks were digested with proteinase K, hybridized with the labeled probe (1 μg/ml) at 60°C for 20 h, and washed with 2× SSC and 0.1× SSC at 47°C. Hybridization signals were visualized as a blue-black NBT/BCIP deposit against a methyl green nuclear counterstain.

Tissue Culture and Transfection

Primary human ECs (Clonetics) were grown to confluence in EGM complete medium (Clonetics), trypsinized, and resuspended in culture media to a density of 8×10^6/ml. Cells (250 μl) and plasmid DNA (10 μg) were combined into a 0.4-cm cuvette and electroporation was conducted at 0.22 Kv, 0.95 μF for 50–60 ms in a Gene Pulser II unit (BioRad). Cells were resuspended in culture media, plated in flasks or 6-well dishes with glass coverslips, and returned to a CO_2 incubator for various length of time before observation and further sample processing.

Reverse Transcription (RT)-PCR

Cells were transfected with EGFP-C3 with or without Ges, and plated in T-75 flasks as described above. After 24 h of incubation, they were washed twice with HBSS, lysed with TRIzol reagent (Life Technologies), and total RNA was isolated according to the manufacturer's protocol. The purified RNA was then treated with RNase-free DNase set/off column kit

(Qiagen) to degrade contaminating genomic and plasmid DNA. cDNA was synthesized from 2.5 μg purified DNA-free total RNA and 0.5 μg oligo $(dT)_{12}$ using Superscript II enzyme (Life Technologies) according to the manufacturer's protocol. As RT minus negative controls to assess contaminating plasmid DNA (EGFP-C3/Ges) in the RNA samples, equal amounts of total RNA used for the RT reactions were subjected to the same RT treatment in the absence of oligo $(dT)_{12}$ and Superscript II. After termination of the RT reaction by incubation at 70°C for 15 min, the samples were incubated with 2 U RNase H at 37°C for 20 min to degrade RNA.

Pairs of RT-plus and RT-minus PCR reactions were conducted using Advantage-HF 2 Polymerase mix (CLONTECH Laboratories, Inc.). Gene-specific primers for Ges (5′ ATGACACTCAACACCGAGCAGGAA 3′ and 5′ TCAGAGCACGGCCAGATTGTGGCA 3′) were chosen from two different exons (genomic sequence data not shown) to amplify an 897-bp Ges cDNA fragment. Gene-specific primers for housekeeping gene hypoxanthine guanine phosphoribosyl transferase (HPRT; 5′ GGCGTCGTGATTAGTGATGA 3′ and 5′ TCACCAGCAAGCTTGCGAC 3′) were chosen across intron–exon boundaries to amplify a 479-bp HPRT cDNA fragment. PCR reactions were conducted for 35 cycles at annealing temperature of 60°C for Ges and 55°C for HPRT. Equal amounts of each reaction were run on a 0.8% agarose gel.

Western Blot Analysis

Cells were transfected with EGFP-C3 with or without Ges, and plated in T-75 flasks as described above. After 24 h of incubation, they were washed twice with PBS and lysed in mammalian protein extraction reagent (M-PER; Pierce Chemical Co.) containing protease inhibitors. Two sets of cell lysates (10 μg total protein/sample) were run on one 10% polyacrylamide SDS gel and transferred to a nitrocellulose membrane. The membrane was cut in half, with one half probed with polyclonal antibody against green fluorescent protein (GFP; Molecular Probes), and the other half probed with polyclonal antibody against peptide KPSPAPDDWSSE from the hypervariable Ges NH_2-terminal domain. Western blots were developed using a chemiluminescence Western blotting kit (Boehringer) according to the manufacturer's protocol.

Fluorescence Microscopy

For regular fluorescence microscopy, coverslips were fixed for 15 min in 4% paraformaldehyde with PBS wash before and after the treatment, and mounted onto a slide. For visualization of actin, the coverslips were subjected to the following sequential treatment: 15 min fixation in 4% paraformaldehyde, 10 min permeabilization in 0.2% Triton-X 100, 10-min blocking in 1% BSA, and 20-min staining with rhodamine phalloidin conjugate (Molecular Probes), with PBS wash before and after each treatment, and then mounted onto slides. For vinculin visualization, the coverslips were sequentially incubated in mouse anti–human vinculin and goat anti–mouse IgG conjugated with TRITC each (Sigma Chemical Co.) for 30 min, with PBS wash before and after each treatment, and then mounted onto slides. Images were taken onto slide films either directly from culture with an Olympus IX-70 inverted fluorescence microscope or from the slides using an Olympus AX-70 fluorescence microscope, digitized in a Nikon LS-1000 35-mm Slide Scanner, and image contrast was adjusted using Adobe Photoshop.

Results

Identification of Ges, a Human GTPase of the RGK Family

A search of the Incyte EST database with the COOH-terminal motif (KSKSCHN/DLA/SVL) yielded one clone (Incyte 724666) with partial sequence. Subsequent screening of a human lymph node λ phage library resulted in three independent clones, each containing the full coding sequence, as judged by comparison with other RGKs, open reading frames, the location of an ATG translation start codon, and an upstream stop codon. The full-length sequence is composed of 897 bp open reading frame, and 280 and 794 bp of 5′ and 3′ untranslated regions, respectively. The poly-A tails of the four clones (Incyte 724666 and the three clones from library screening) were added at different positions 11–25 bp downstream of two polyadenylation signals (AATAAA) positioned 137 bp apart (Fig. 1 a).

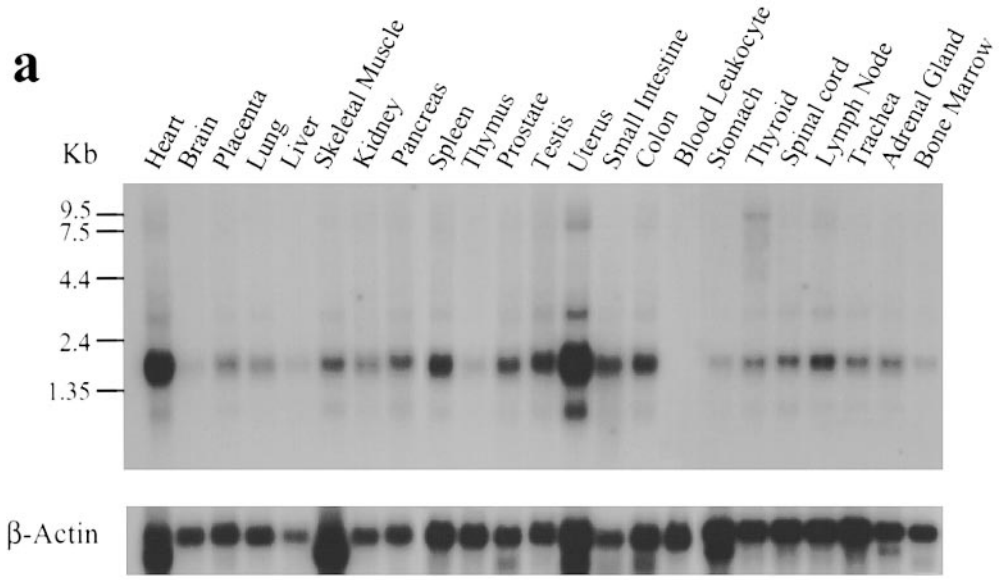
The deduced protein, Ges, contains 298 amino acids with a calculated molecular weight of 32,946 D. It shares not only high sequence similarity with Rad (43.8%), Gem (44.1%), and mRem (87.9%), but also all of the unique structural features conserved in the RGKs, which include the unique G domains, extended NH_2- and COOH-terminal domains beyond the Ras-like core, a putative calmodulin-binding domain, and the COOH-terminal motif (Fig. 1 b). Ges sequence diverges from other RGK members in four regions (the hypervariable NH_2-terminal domain, a variable region in the COOH-terminal domain, and the putative Switch I and Switch II domains that align with the switch domains in Ras), which likely confer functional specificity within this GTPase family (Fig. 1 b). Consistent with predictions from Ges primary sequence, in vitro assays confirmed that recombinant Ges bound GTP and GDP, exhibited low intrinsic GTPase activity, and interacted with calmodulin in Ca^{2+}-dependent manner (data not shown).

Expression Profile of Ges

Northern blot analysis of human multiple tissue samples revealed that Ges was expressed in a wide variety of tissues, most abundantly in uterus and heart, and rarely in brain, liver, bone marrow, and thymus (Fig. 2 a). To elucidate the specific cell types that express Ges in vivo, ISH was conducted on a number of human tissues, including heart, uterus, brain, liver, prostate, lung, pancreas, colon, and breast. These surveys revealed that Ges was expressed predominantly at high levels in the endothelium lining the blood vessels in uterus (Fig. 2 b) and heart, rather low levels in vessels from tissues such as colon and breast, and absent in vessels from brain and liver (data not shown). Ges expression was also detected at various levels in cells with apparent secretory functions, including islets of Langerhans in the pancreas, lobule/duct epithelium in the breast, bile duct epithelium in the liver, surface epithelium in the endometrial glands in the uterus, colon mucosa, and acinar cells in the pancreas and the prostate (data not shown).

Ges Is Sufficient to Promote EC Sprouting in the Absence of ECM/Angiogenic Growth Factor Stimulation

To explore Ges function in ECs, we studied the effect of its overexpression in cultured primary human umbilical cord artery ECs (HUAECs), which, under normal culture conditions, express Ges at extremely low levels, but upon transfection with EGFP-C3/Ges exhibit elevated level of Ges expression for both mRNA (assessed by RT-PCR) and GFP-Ges fusion protein (assessed by Western blot; Fig. 3 a). Approximately two to three hours after transfection with the EGFP-C3 vector, transfected HUAECs started to turn green due to the expression of GFP encoded in the vector, and thus could be observed under a fluorescence microscope. In comparison with cells transfected with the empty vector that maintained round/oval



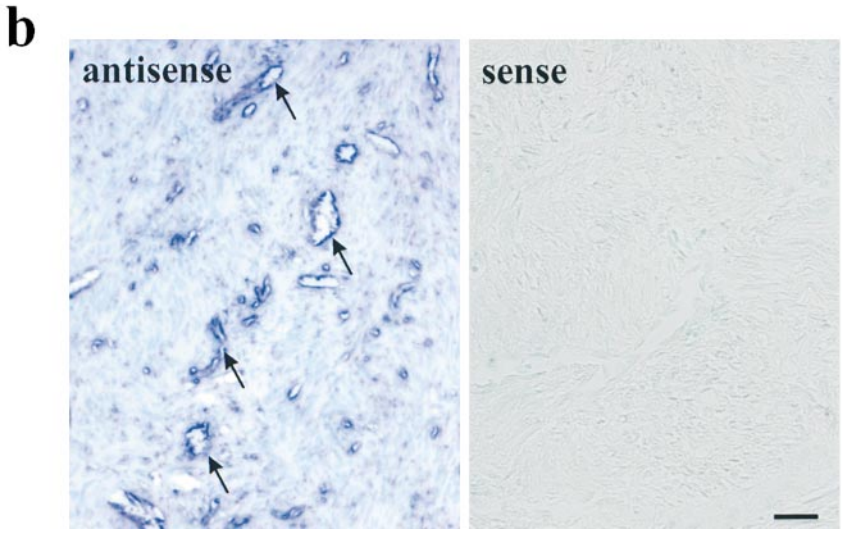


Figure 2. Expression profile of Ges. a, Multiple tissue Northern blot. Human multiple tissue blots (2 μg mRNA/lane; CLONTECH Laboratories, Inc.) were hybridized with a ^{32}P-labeled Ges probe, then stripped and probed for β-actin as described by the manufacturer. This blot is representative of several repeats using different Ges probes. b, ISH of human uterus. Tissue sections from the same series were hybridized with digoxigenin-labeled sense or antisense probe. Ges hybridization signal is visualized as blue-black NBT/BCIP deposit against a methyl green nuclear counterstain. Bar, 40 μm.

shape, >90% of the cells transfected with EGFP-C3/Ges quickly developed cytoplasmic processes and took on significantly elongated or dendritic-like morphology (Fig. 3 b). The initiation of this morphology change could be observed within four hours after transfection and the sprouts reached maximum length within 24 h. This potent ability to promote EC sprouting was observed when GFP and Ges were expressed separately (via EGFP-pIRES vector that contains two ribosome entry sites, one before each of the coding region of GFP and the inserted gene of interest), as well as fusion protein GFP-Ges (via EGFP-C3 vector). Identical results were also observed for all additional types of ECs studied, which include primary human umbilical cord vein ECs (HUVECs; Fig. 3 c), primary human coronary artery ECs (HCAECs; Fig. 3 d), and primary human pulmonary artery ECs (HPAECs; Fig. 3 e). In contrast, when other RGK member Rad was overexpressed in muscle and fat cells (Moyers et al., 1996), or Gem was overexpressed in CV-1, NTERA-2, and ECs (Maguire et al., 1994; Vanhove et al., 1997; and Fig. 3 b), significant cell morphology change was not observed.

Ges shares 87.9% sequence similarity with mRem, a mouse protein with unknown function. Since most of the 33 amino acid substitutions between Ges and mRem are nonconservative, in particular, eight of which involve Pro, a residue that significantly alters protein conformation; and also since most of these amino acid substitutions fall within the four variable regions that define different members within the RGK family (Fig. 1 b), it was unclear whether Ges is the human orthologue of mRem. When mRem was transfected into primary human ECs, it promoted very similar sprouting morphology (Fig. 3 b), suggesting that mRem has the potential to engage in the same signaling pathway.

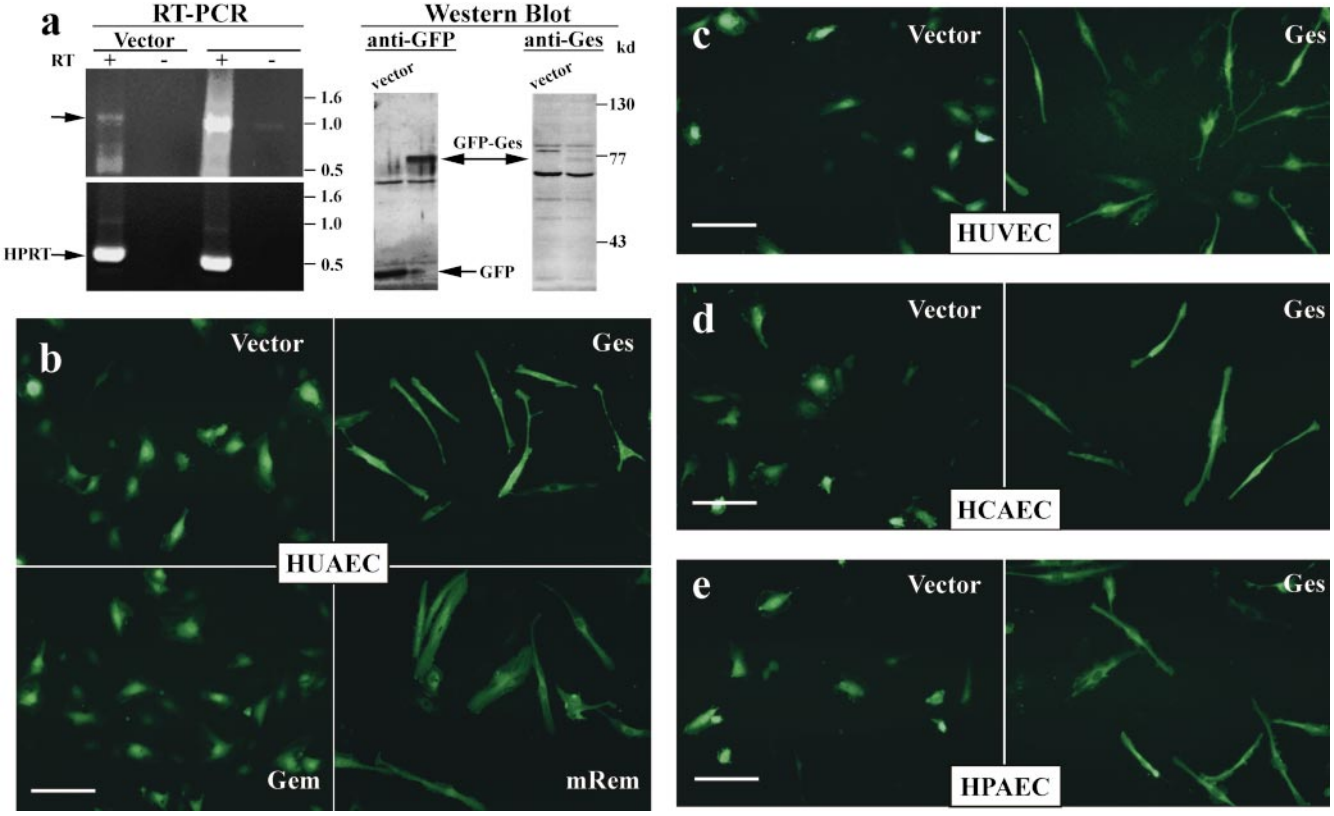


Figure 3. Ges promotes EC sprouting. a, Overexpression of Ges mRNA and protein in HUAECs transfected with EGFP-C3/Ges. Primary HUAECs were transfected with EGFP-C3 with or without Ges. After 24 h of incubation, cells were lysed in corresponding buffers to isolate total RNA for RT-PCR or total protein for Western blot as described in Materials and Methods. In the RT-PCR panel, the faint band in the RT− lane of Ges transfection is due to the incomplete DNase digestion of the plasmid DNA EGFP-C3/Ges. In the Western blot panel, endogenous Ges, which migrates at ~40 kD on the gel, could not be detected by the Ges antibody. b, HUAECs were transfected with EGFP-C3, EGFP-C3/Ges, EGFP-C3/mRem, or EGFP-pIRES 2/Gem and incubated on glass coverslips for 24 h before photography. c–e, HUVECs, HCAECs, or HPAEC transfected with EGFP-C3 with or without Ges the same way as described for b. Bars, 100 μm.

Ges Promotes Cytoskeleton Reorganization in ECs

To investigate possible changes in the cytoskeleton that coincide with Ges-induced EC sprouting, actin filaments were examined by rhodamine phalloidin staining. In HUAECs transfected with the empty vector (Fig. 4 a) or normal untransfected cells (Fig. 4 c), actin stress fibers were arranged into star-like criss-crossing clusters and were oriented in all directions across the cell body. Whereas in cells transfected with Ges (Fig. 4, b and c), actin was reorganized into bundles of long filaments parallel to each other along the axis of the elongated cells, and was also concentrated at the cell periphery. This characteristic change of the actin architecture has been observed previously, when ECs receive combined signals from angiogenic growth factors and ECM proteins in vitro (Grant et al., 1991; Schenk et al., 1999). Concomitant with the rearrangement of the actin fibers, vinculin staining revealed noticeable reduction in the sizes of focal adhesion complexes in the sprouting cells (Fig. 4 d), indicating decreased cell attachment to the culture surface.

Ges Function Is Necessary for Matrigel-induced EC Sprouting

To explore the physiological relevance of the observed Ges-induced EC morphology change, dominant negative mutant Ges^{T94N} was developed based on analogy with other Ras-like GTPases, and was used to examine the involvement of Ges in Matrigel-induced EC sprouting. Derived from murine sarcoma, Matrigel is a complex mixture of ECM proteins and growth factors, and exhibits potent ability to promote EC sprouting/rudimentary tube formation in vitro. When ECs are plated on Matrigel, they quickly lose the cobblestone morphology and begin developing cytoplasmic extensions to reorganize themselves first into chains of cells, and later into rudimentary tube-like structures. Since this process bears resemblance to angiogenesis, it is used frequently to study aspects of angiogenesis in vitro (Belotti et al., 1996; Baatout, 1997; Isaji et al., 1997; Sheibani et al., 1997; Yatsunami et al., 1997; Cockerill et al., 1998; Hisa et al., 1998; Lelkes et al., 1998; Oikawa et al., 1998; Pipili-Synetos et al., 1998; Riccioni et al., 1998; Thaloor et al., 1998; Gho et al., 1999; Lamszus et al., 1999; Malinda et al., 1999; Ribatti et al., 1999). The conserved T^{94} in Ges (corresponding to S^{17} in Ras) is predicted to mediate Mg^{2+} coordination and, therefore, guanine nucleotide binding. Mutation of this residue is expected to lock the protein in the nucleotide-free conformation with high affinity for its guanine nucleotide exchange factor (GEF; Pan and Wessling-Resnick, 1998). Cognate mutants of

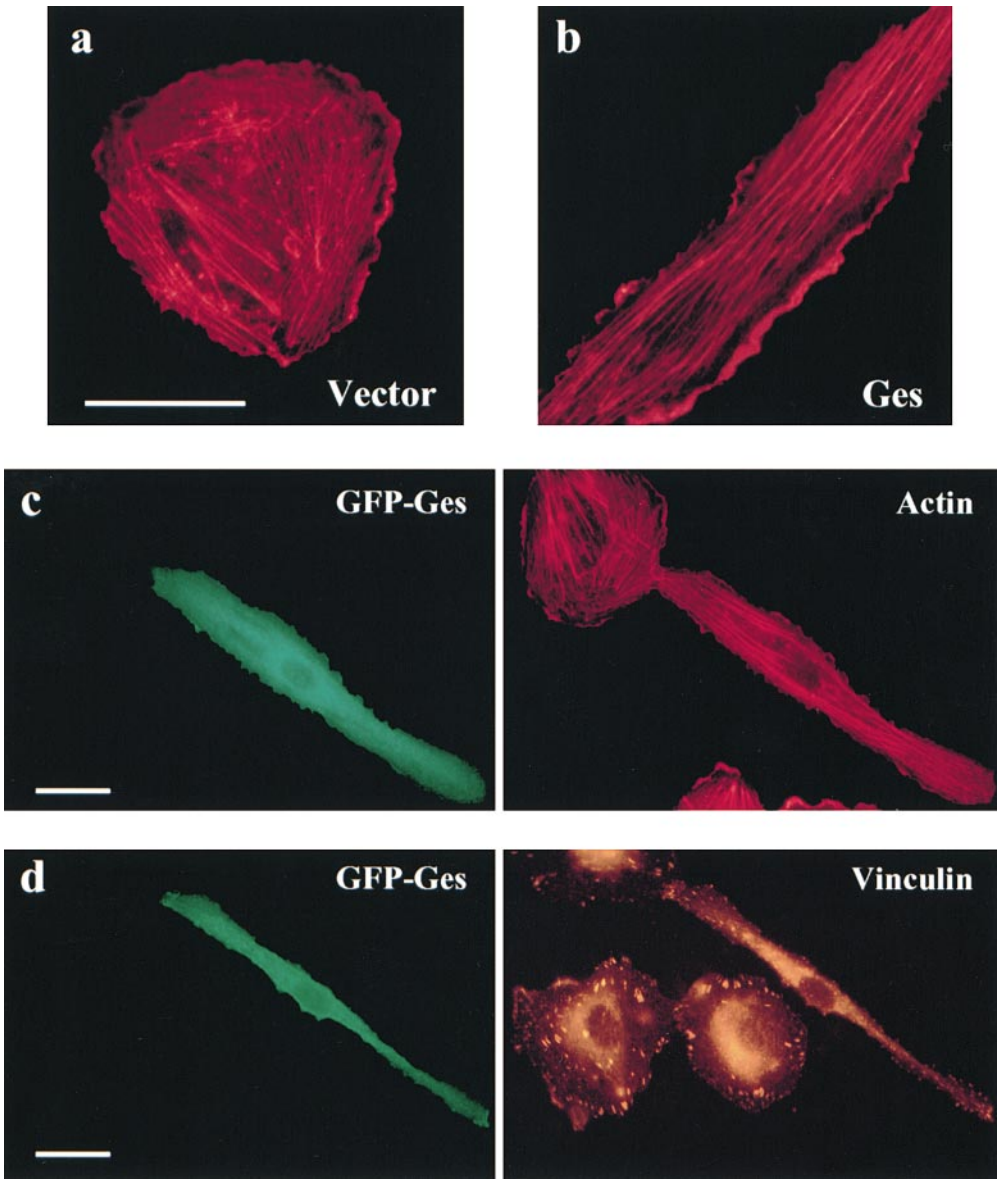


Figure 4. Ges promotes cytoskeleton reorganization in ECs. HUAECs were transfected with EGFP-C3 (a) or EGFP-C3/Ges (b–d), plated on glass coverslips, and incubated for 24 h. The coverslips were then fixed and processed for actin (a–c) and vinculin (d) visualization. Bars, 30 μM.

Ges^{T94N} have been used routinely as the dominant negative mutants in functional studies of many GTPases due to their ability to sequester specific GEFs and thus to prevent the activation of their endogenous wild-type counterparts (Feig, 1999). EGFP-C3 with or without Ges or Ges^{T94N} was transfected into ECs through electroporation. The mixture of transfected (~30%) and untransfected (~70%) cells was then plated on Matrigel and observed over time in culture. In comparison with cells transfected with the empty vector, which exhibited comparable level of sprouting to the untransfected cells, Matrigel-induced EC sprouting was strongly enhanced by wild-type Ges, but blocked completely by Ges^{T94N} (Fig. 5). In contrast, cognate mutant of the closely related RGK member Gem^{S89N} failed to block EC sprouting on Matrigel (Fig. 5 a). This specific inhibition exerted by Ges^{T94N} indicates that endogenous Ges is essential for EC sprouting induced by Matrigel.

Discussion

We report the identification of Ges, a human GTPase of the RGK family, for which expression is both sufficient and necessary to promote EC morphology change that mimics cell sprouting during angiogenesis in vivo. When ECs are cultured on glass surface in the absence of growth factor stimulation, they normally exhibit cobblestone morphology. Cell sprouting was observed only when they were plated on, or embedded in, gels of various ECM proteins and stimulated with angiogenic factors, such as vascular endothelial growth factor, angiopoietin 1 (Koblizek et al., 1998), bFGF (Montesano et al., 1986; Gualandris et al., 1996; Schenk et al., 1999), and PMA (Montesano and Orci, 1985). The potent ability of Ges to promote EC sprouting, even when the cells are cultured on glass in the absence of growth factor stimulation, indicates that Ges functions downstream of, and therefore substitutes for, combined signals from angiogenic growth factors and ECM mole-

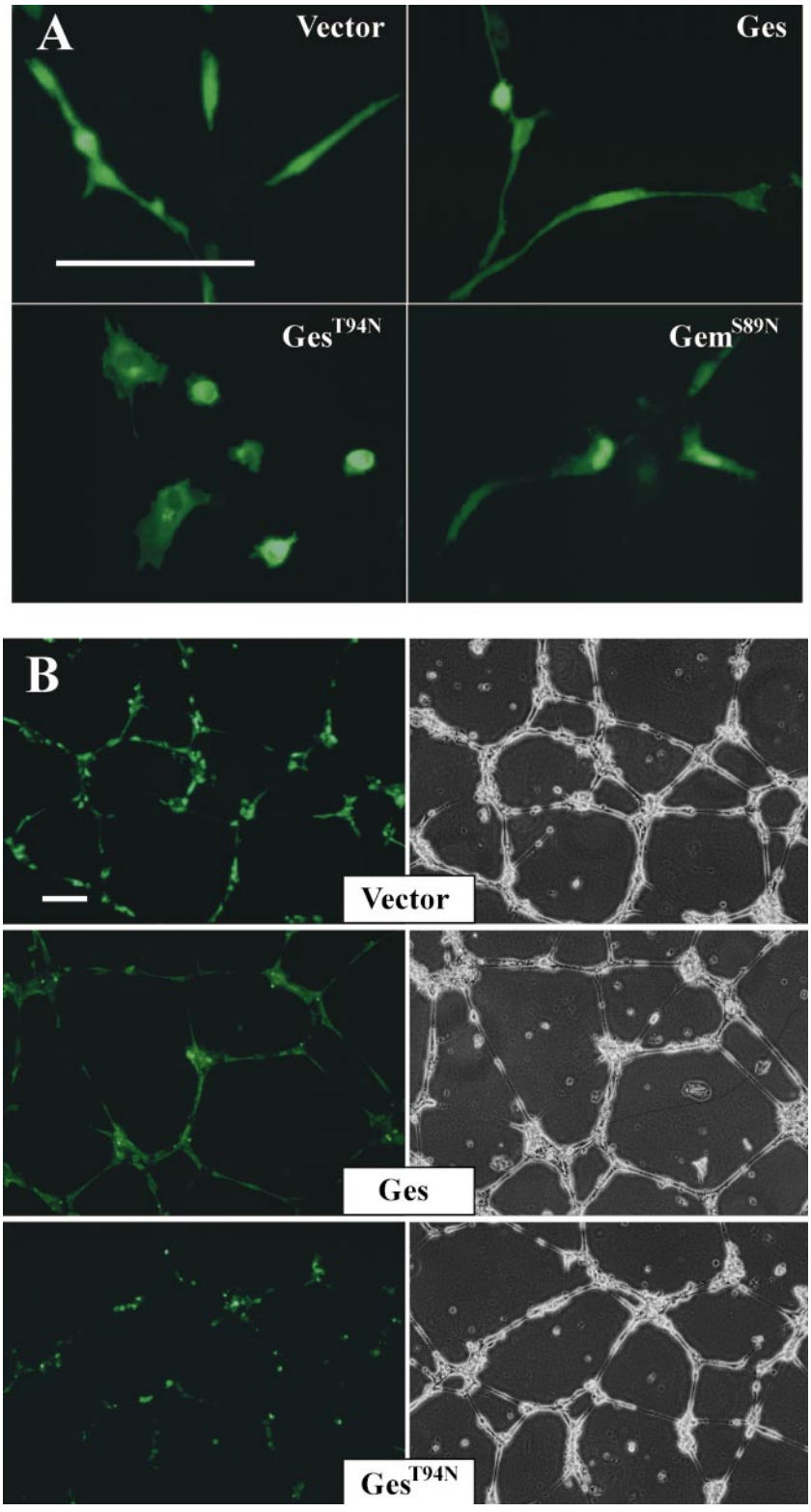


Figure 5. Ges^{T94N} inhibits Matrigel-induced EC sprouting. Primary HUAECs were transfected with EGFP-C3 with or without Ges, Ges^{T94N}, or Gem^{S89N} through electroporation. The mixture of transfected and untransfected cells was plated on Matrigel-coated plates and incubated for 5 (a) or 24 h (b) before photography. The phase-contrast micrographs in b show the networks formed by the mixture of untransfected and transfected cells. The fluorescence micrographs show the morphology and involvement in network formation of the transfected cells only. Bars, 150 μM.

cules. Its low intrinsic GTPase activity likely contributes to the accumulation of the newly synthesized Ges in the GTP-bound form that promotes cell sprouting without the upstream activating signal. The crucial role of Ges in EC sprouting is further supported by the observation that Matrigel-induced EC sprouting was blocked effectively by the dominant negative mutant Ges^{T94N}, which presumably prevents the activation of the endogenous Ges through sequestration of its GEF. Together, these findings suggest that Ges acts as a molecular switch essential for the inte-

gration of growth factor/ECM signals and coupling such signals to downstream events, including actin cytoskeleton reorganization and EC sprouting.

Rho, Rac, and CDC42 constitute a family of ubiquitously expressed Ras-like GTPases that coordinately regulate the functions of the actin cytoskeleton, as well as many other cellular activities (Tapon and Hall, 1997; Hall, 1998). In fibroblasts, Rho, Rac, and CDC42 each promote distinctive actin cytoskeleton/cell shape changes, characterized as stress fiber/focal adhesion, lamellipodia/membrane ruffle, and filopodia, respectively. In other cells, effects exerted by each of the Rho family members are modified by parameters characteristic of the specific cell types. In comparison to Rho/Rac/CDC42, Ges clearly promotes distinctive EC morphology change and actin cytoskeleton reorganization, exhibits rather restricted in vivo expression pattern, and its ability to promote cell morphology change in vitro is also limited to a subset of cell types. Among all the non-ECs and cell lines studied, Ges promotes significant morphology changes in primary human bladder smooth muscle cells and coronary artery smooth muscle cells, ECV 304, and fibroblast cell lines IMR-90 and NIH3T3. It had no obvious effect on cell morphology in carcinoma cell lines (HeLa, A-431 and A549), myeloma cell lines (G-361 and DU-145), and a neuronal precursor cell line (NT-2; data not shown). In depth studies are needed to determine how the Ges signaling pathway operates in cell type-specific manner, whether and how it cross-talks with the Rho GTPases, and whether other members of the RGK family exert their observed function also through regulation of the actin cytoskeleton organization.

Angiogenesis, the process of new blood vessel formation, is characterized by a series of events including increased vascular permeability, proteolytic degradation of basement membrane, chemotactic sprouting, migration and proliferation of ECs, lumen formation, and functional maturation of the endothelium. A large array of pro- and antiangiogenic factors, as well as ECM molecules, has been identified to regulate different steps of angiogenesis by transmitting signals simultaneously across EC membrane through cell surface receptors and integrins, respectively (Klagsbrun and D'Amore, 1991). Nonetheless, very little is known about the intracellular signaling pathways that link signals at the cell surface to the angiogenic phenotypes. The correlation between predominant Ges expression in the uterus endothelium (where there is active angiogenesis) and the potent ability of Ges to promote EC sprouting (one of the hallmarks of angiogenesis) strongly suggests a crucial involvement of Ges in blood vessel formation. Thus, further dissection of the Ges pathway should provide much insight into the molecular mechanism of angiogenesis and its regulation.

We thank Dr. Paul Elvin, Dr. Hans Winkler, Dr. Richard Clark, and Dr. Steve McClain for scientific advice and discussions; Dr. Dimuthu deSilva for the Gem^{S89N} construct; Lee Hirata and Patricia Sherman for sequencing support; Yockey Courtland for help in bioinformatic search; and Katie Kron, Jason Butler, and Amanda Brosius for other technical assistance.

Submitted: 31 January 2000
Revised: 18 April 2000
Accepted: 21 April 2000

References

Baatout, S. 1997. Endothelial differentiation using Matrigel. *Anticancer Res.* 17: 451–455.

Belotti, D., V. Vergani, T. Drudis, P. Borsotti, M.R. Pitelli, G. Viale, R. Giavazzi, and G. Taraboletti. 1996. The microtubule-affecting drug paclitaxel has antiangiogenic activity. *Clin. Cancer Res.* 2:1843–1849.

Bilan, P.J., J.S. Moyers, and C.R. Kahn. 1998. The Ras-related protein Rad associates with the cytoskeleton in a non-lipid-dependent manner. *Exp. Cell Res.* 242:391–400.

Bourne, H.R., D.A. Sanders, and F. McCormick. 1991. The GTPase superfamily: conserved structure and molecular mechanism. *Nature.* 349:117–127.

Cockerill, G.W., L. Varcoe, G.T. Meyer, M.A. Vadas, and J.R. Gamble. 1998. Early events in angiogenesis: cloning an alpha-prolyl 4-hydroxylase-like gene. *Int. J. Oncol.* 13:595–600.

Cohen, L., R. Mohr, Y.Y. Chen, M. Huang, R. Kato, D. Dorin, F. Tamanoi, A. Goga, D. Afar, N. Rosenberg, and O. Witte. 1994. Transcriptional activation of a ras-like gene (kir) by oncogenic tyrosine kinases. *Proc. Natl. Acad. Sci. USA.* 91:12448–12452.

Del Villar, K., D. Dorin, I. Sattler, J. Urano, P. Poullet, N. Robinson, H. Mitsuzawa, and F. Tamanoi. 1996. C-terminal motifs found in Ras-superfamily G-proteins: CAAX and C-seven motifs. *Biochem. Soc. Trans.* 24:709–713.

Dorin, D., L. Cohen, K.D. Villar, P. Poullet, R. Mohr, M. Whiteway, O. Witte, and F. Tamanoi. 1995. Kir, a novel Ras-family G-protein, induces invasive pseudohyphal growth in *Saccharomyces cerevisiae. Oncogene.* 11:2267–2271.

Feig, L.A. 1999. Tools of the trade: use of dominant-inhibitory mutants of Ras-family GTPases. *Nat. Cell Biol.* 1:E25–E27.

Finlin, B.S., and D.A. Andres. 1997. Rem is a new member of the Rad- and Gem/Kir Ras-related GTP-binding protein family repressed by lipopolysaccharide stimulation. *J. Biol. Chem.* 272:21982–21988.

Fischer, R., Y. Wei, J. Anagli, and M.W. Berchtold. 1996. Calmodulin binds to and inhibits GTP binding of the ras-like GTPase Kir/Gem. *J. Biol. Chem.* 271:25067–25070.

Gho, Y.S., H.K. Kleinman, and G. Sosne. 1999. Angiogenic activity of human soluble intercellular adhesion molecule-1. *Cancer Res.* 59:5128–5132.

Grant, D.S., P.I. Lelkes, K. Fukuda, and H.K. Kleinman. 1991. Intracellular mechanisms involved in basement membrane induced blood vessel differentiation in vitro. *In Vitro Cell. Dev. Biol.* 27A:327–336.

Gualandris, A., M. Rusnati, M. Belleri, E.E. Nelli, M. Bastaki, M.P. Molinari-Tosatti, F. Bonardi, S. Parolini, A. Albini, L. Morbidelli, et al. 1996. Basic fibroblast growth factor overexpression in endothelial cells: an autocrine mechanism for angiogenesis and angioproliferative diseases. *Cell Growth Differ.* 7:147–160.

Hall, A. 1998. Rho GTPases and the actin cytoskeleton. *Science.* 279:509–514.

Hisa, T., Y. Kimura, K. Takada, F. Suzuki, and M. Takigawa. 1998. Shikonin, an ingredient of *Lithospermum erythrorhizon*, inhibits angiogenesis in vivo and in vitro. *Anticancer Res.* 18:783–790.

Isaji, M., H. Miyata, Y. Ajisawa, Y. Takehana, and N. Yoshimura. 1997. Tranilast inhibits the proliferation, chemotaxis and tube formation of human microvascular endothelial cells in vitro and angiogenesis in vivo. *Br. J. Pharmacol.* 122:1061–1066.

Klagsbrun, M., and P.A. D'Amore. 1991. Regulators of angiogenesis. *Annu. Rev. Physiol.* 53:217–239.

Koblizek, T.I., C. Weiss, G.D. Yancopoulos, U. Deutsch, and W. Risau. 1998. Angiopoietin-1 induces sprouting angiogenesis in vitro. *Curr. Biol.* 8:529–532.

Lamszus, K., N.O. Schmidt, S. Ergun, and M. Westphal. 1999. Isolation and culture of human neuromicrovascular endothelial cells for the study of angiogenesis in vitro. *J. Neurosci. Res.* 55:370–381.

Lelkes, P.I., K.L. Hahn, D.A. Sukovich, S. Karmiol, and D.H. Schmidt. 1998. On the possible role of reactive oxygen species in angiogenesis. *Adv. Exp. Med. Biol.* 454:295–310.

Maguire, J., T. Santoro, P. Jensen, U. Siebenlist, J. Yewdell, and K. Kelly. 1994. Gem: an induced, immediate early protein belonging to the Ras family. *Science.* 265:241–244.

Malinda, K.M., L. Ponce, H.K. Kleinman, L.M. Shackelton, and A.J. Millis. 1999. Gp38k, a protein synthesized by vascular smooth muscle cells, stimulates directional migration of human umbilical vein endothelial cells. *Exp. Cell. Res.* 250:168–173.

Montesano, R., and L. Orci. 1985. Tumor-promoting phorbol esters induce angiogenesis in vitro. *Cell.* 42:469–477.

Montesano, R., J.D. Vassalli, A. Baird, R. Guillemin, and R. Orci. 1986. Basic fibroblast growth factor induces angiogenesis in vitro. *Proc. Natl. Acad. Sci. USA.* 83:7297–7301.

Moyers, J.S., P.J. Bilan, C. Reynet, and C.R. Kahn. 1996. Overexpression of Rad inhibits glucose uptake in cultured muscle and fat cells. *J. Biol. Chem.* 271:23111–23116.

Moyers, J.S., P.J. Bilan, J. Zhu, and C.R. Kahn. 1997. Rad and Rad-related GTPases interact with calmodulin and calmodulin-dependent protein kinase II. *J. Biol. Chem.* 272:11832–11839.

Oikawa, T., T. Sasaki, M. Nakamura, M. Shimamura, N. Tanahashi, S. Omura. and K. Tanaka. 1998. The proteasome is involved in angiogenesis. *Biochem. Biophys. Res. Commun.* 246:243–248.

Pan, J.Y., and M. Wessling-Resnick. 1998. GEF-mediated GDP/GTP exchange by monomeric GTPases: a regulatory role for Mg^{2+}? *Bioessays.* 20:516–521.

Pipili-Synetos, E., E. Papadimitriou, and M.E. Maragoudakis. 1998. Evidence

that platelets promote tube formation by endothelial cells on matrigel. *Br. J. Pharmacol*. 125:1252–1257.
Reynet, C., and C.R. Kahn. 1993. Rad: a member of the Ras family overexpressed in muscle of type II diabetic humans. *Science*. 262:1441–1444.
Ribatti, D., M. Presta, A. Vacca, R. Ria, R. Giuliani, P. Dell'Era, B. Nico, L. Roncali, and F. Dammacco. 1999. Human erythropoietin induces a pro-angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo. *Blood*. 93:2627–2636.
Riccioni, T., C. Cirielli, X. Wang, A. Passaniti, and M.C. Capogrossi. 1998. Adenovirus-mediated wild-type p53 overexpression inhibits endothelial cell differentiation in vitro and angiogenesis in vivo. *Gene Ther*. 5:747–754.
Schenk, S., R. Chiquet-Ehrismann, and E.J. Battegay. 1999. The fibrinogen globe of tenascin-C promotes basic fibroblast growth factor-induced endothelial cell elongation. *Mol. Biol. Cell*. 10:2933–2943.
Sheibani, N., P.J. Newman, and W.A. Frazier. 1997. Thrombospondin-1, a natural inhibitor of angiogenesis, regulates platelet–endothelial cell adhesion molecule-1 expression and endothelial cell morphogenesis. *Mol. Biol. Cell*. 8:1329–1341.
Tapon, N., and A. Hall. 1997. Rho, Rac and Cdc42 GTPases regulate the organization of the actin cytoskeleton. *Curr. Opin. Cell Biol*. 9:86–92.
Thaloor, D., A.K. Singh, G.S. Sidhu, P.V. Prasad, H.K. Kleinman, and R.K. Maheshwari. 1998. Inhibition of angiogenic differentiation of human umbilical vein endothelial cells by curcumin. *Cell Growth Differ*. 9:305–312.
Vanhove, B., R. Hofer-Warbinek, A. Kapetanopoulos, E. Hofer, F.H. Bach, and R. Martin. 1997. Gem, a GTP-binding protein from mitogen-stimulated T cells, is induced in endothelial cells upon activation by inflammatory cytokines. *Endothelium*. 5:51–61.
Yatsunami, J., N. Turuta, K. Wakamatsu, N. Hara, and S. Hayashi. 1997. Clarithromycin is a potent inhibitor of tumor-induced angiogenesis. *Res. Exp. Med*. 197:189–197.
Zhu, J., C. Reynet, J.S. Caldwell, and C.R. Kahn. 1995. Characterization of Rad, a new member of Ras/GTPase superfamily, and its regulation by a unique GTPase-activating protein (GAP)-like activity. *J. Biol. Chem*. 270: 4805–4812.