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

Julie Y. Pan,* William E. Fieles,* Anne M. White,‡ Mark M. Egerton,§ and David S. Silberstein*

*Enabling Science and Technology-Biology, AstraZeneca Pharmaceuticals, Wilmington, Delaware 19850-5437; and ‡Department of Cancer and Infection, AstraZeneca Pharmaceuticals, Alderley Park, Macclesfield, Cheshire, SK10 4TG United Kingdom

Introduction

Rad, Gem/Kir, and mRem (RGK) 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), 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 tissues of mice injected with lipopolysaccharide (Finlin and Andres, 1997). 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), 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; K ir 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 A ndres, 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 (G12 in Ras) is replaced by Pro or Gln, and the entire conserved G2 domain (DTAGQ 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 Ca2+-dependent manner, suggesting their involvement in Ca2+ signaling (Fischer et al., 1996; Moyer et al., 1997). A conserved 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 Ca2+-dependent manner, suggesting their involvement in Ca2+ signaling (Fischer et al., 1996; Moyer et al., 1997). A conserved
COOH-terminal domain (KSKCHN/DLA/SVL): initially speculated to be a novel isoprenylation motif (R eynet and K ahn, 1993; M aguire et al., 1994), but later shown not to be modified by isoprenylation (D el V illar et al., 1996; B ilan 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 (M oyers et al., 1996), that Gem overexpression significantly reduced the number of selectable colonies in NIH 3T3 (M aguire et al., 1994; V anhove et al., 1997), and that Kir overexpression induced invasive pseudohyphal growth in Saccharomyces cerevisiae (D orin 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 G es (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.

Materials and Methods

Database Search and Library Screening

Sequence similarity search of a proprietary expressed sequence tag (EST) database (LIF ESEQ; Incyte Pharmaceuticals) was conducted using the conserved COOH-terminal motif in the RGK family (KSKCHN/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-y-old female with osteoarthritis. A 32P-labeled probe corresponding to nucleotides 1,064-1,277 was generated by PCR for library screening. Approximately 600,000 recombinant plasmids from a human lymph node gt10 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. A 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 32P-labeled Ges probe (specific activity ~ 6.0 × 106 cpm/µg), washed (20 min/wash) twice at room temperature with 2 × SSC, 0.05% SDS, twice at 50°C with 0.1 × SSC, 0.01% SDS, and exposed on X-ray film at -70°C for 1 wk. The film 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 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 × 106/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 CO2 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. A fter 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/aff column kit.
Gene-specific primers for Ges (5′-TGAGACTCTCAACAAGCCAGCAGGAA3′ and 5′-TCAAGACCGCGACTGTTGGCAGC3′) 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′-GGGCCCTGTAGATTGATGA3′ and 5′-TCAACCAGCAGGCTTGGCA3′) 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. A few hours after 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 monoclonal antibody against beta actin (Sigma Chemical Co.) for 1 h, washed and then reacted with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG (Molecular Probes), with PBS wash before and after each treatment, and then mounted onto slides. For vinculin visualization, the cover slips were subjected to the following sequential treatment: 15 min fixation in 4% paraformaldehyde with PBS wash before and after the treatment, and then reacted with anti–mouse IgG conjugated with TRITC each (Sigma Chemical Co.) for 1 h, washed and then reacted with HRP conjugated with anti-rabbit or anti-mouse IgG (Molecular Probes), with PBS wash before and after each treatment, and then mounted onto slides. For visualization of actin, the coverslips were subjected to the following sequential treatment: 15 min fixation in 4% paraformaldehyde with PBS wash before and after the treatment, and then reacted with anti–mouse IgG conjugated with TRITC each (Sigma Chemical Co.) for 1 h, washed and then reacted with HRP conjugated with anti-rabbit or anti-mouse IgG (Molecular Probes), with PBS wash before and after each treatment, and then mounted onto slides. The slides were dehydrated, cleared, and sealed with a cover slip.

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 (5′-SKSCHN/D/LS/AVL′) 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′ untranscribed 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 (AAATAAA) positioned 137 bp apart (Fig. 1a).

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 NH2- and COOH-terminal domains beyond the Ras-like core, a putative calmodulin-binding domain, and the COOH-terminal motif (Fig. 1b). Ges sequence diverges from other RGK members in four regions (the hypervariable NH2-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. 1b). 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 Ca2+-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. 2a). 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. 2b) 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 (assayed by RT-PCR) and GFP-Ges fusion protein (assayed by Western blot; Fig. 3a). 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...
shape, >90% of the cells transfected with EGFP-C3/Ges quickly developed cytoplasmic processes and took on significantly elongated or dendritic-like morphology (Fig. 3b). 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. 3c), primary human coronary artery ECs (HCAECs; Fig. 3d), and primary human pulmonary artery ECs (HPAECs; Fig. 3e). 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 (Magueire et al., 1994; Vanhove et al., 1997; and Fig. 3b), 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. 1b), 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. 3b), suggesting that mRem has the potential to engage in the same signaling pathway.
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$^{T94D}$ 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 tubelike 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
Ges 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<sup>T94N</sup> 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<sup>T94N</sup> (Fig. 5). In contrast, cognate mutant of the closely related RGK member Gem<sup>S89N</sup> failed to block EC sprouting on Matrigel (Fig. 5 a). This specific inhibition exerted by Ges<sup>T94N</sup> 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-
molecules. 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 GEsT⁹⁴⁴N, 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-

Figure 5. GEsT⁹⁴⁴N inhibits Matrigel-induced EC sprouting. Primary HUAECs were transfected with EGFP-C3 with or without GEs, GEsT⁹⁴⁴N, or GemS⁸⁹⁹N 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.
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 spraying, 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’A more, 1991). Nonetheless, very little is known about the intracellular signaling pathways that link signals at the cell surface to the angiogenic phenomena. 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.

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