

A Vascular Gene Trap Screen Defines RasGRP3 as an Angiogenesis-Regulated Gene Required for the Endothelial Response to Phorbol Esters

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We identified Ras guanine-releasing protein 3 (RasGRP3) as a guanine exchange factor expressed in blood vessels via an embryonic stem (ES) cell-based gene trap screen to identify novel vascular genes. RasGRP3 is expressed in embryonic blood vessels, down-regulated in mature adult vessels, and reexpressed in newly formed vessels during pregnancy and tumorigenesis. This expression pattern is consistent with an angiogenic function for RasGRP3. Although a loss-of-function mutation in RasGRP3 did not affect viability, RasGRP3 was up-regulated in response to vascular endothelial growth factor (VEGF) stimulation of human umbilical vein endothelial cells, placing RasGRP3 regulation downstream of VEGF signaling. Phorbol esters mimic the second messenger diacylglycerol (DAG) in activating both protein kinase C (PKC) and non-PKC phorbol ester receptors such as RasGRP3. ES cell-derived wild-type blood vessels exposed to phorbol myristate acetate (PMA) underwent extensive aberrant morphogenesis that resulted in the formation of large endothelial sheets rather than properly branched vessels. This response to PMA was completely dependent on the presence of RasGRP3, as mutant vessels were refractory to the treatment. Taken together, these findings show that endothelial RasGRP3 is up-regulated in response to VEGF stimulation and that RasGRP3 functions as an endothelial cell phorbol ester receptor in a pathway whose stimulation perturbs normal angiogenesis. This suggests that RasGRP3 activity may exacerbate vascular complications in diseases characterized by excess DAG, such as diabetes.

Blood vessels provide cells with the nutrients and oxygen necessary for viability by forming a branched vascular network that links to larger vessels, a process called angiogenesis. For proper angiogenesis, endothelial cells must receive a number of extracellular signals and then assemble these cues into a coordinated biological response (for reviews, see references 16, 24, 30, 57, and 79). Vascular endothelial growth factor A (VEGF-A) and its two high-affinity receptors, flk-1 (VEGF receptor 2 [VEGFR-2]) and flt-1 (VEGFR-1), stimulate signal transduction pathways that regulate endothelial cell proliferation, migration, survival, and permeability (for reviews, see references 29, 64, and 72). VEGF-A is a secreted ligand expressed by nonendothelial cells developmentally and during physiological and pathological angiogenesis in the adult. The importance of VEGF-mediated signaling in vascular development has been clearly demonstrated genetically, since mutations in VEGF-A and both receptors lead to vascular perturbations and embryonic death (11, 28, 31, 63). Moreover, recent studies indicate that VEGF-A levels and perhaps spatial availability must also be regulated for blood vessels to form properly (15, 33, 50, 62).

Recently, the intracellular signaling pathways downstream

of VEGF-A in endothelial cells have been elucidated (for a review, see reference 14). Signaling through Ras is one pathway downstream of VEGF stimulation of flk-1 (18, 38, 48). Ras is a key intracellular signaling hub that incorporates a number of upstream signals and then coordinates these signals into specific biological responses (for a review, see reference 65). Biochemically, Ras is a small GTPase that functions as a GDP-GTP-regulated switch. Stimulus-mediated activation of guanine nucleotide exchange factors (GEFs) facilitates GDP-GTP exchange and promotes the formation of active Ras-GTP (54), whereas GTPase-activating proteins (GAPs) promote formation of inactive, GDP-bound Ras (6). VEGF stimulation of endothelial cells increases the amount of activated Ras (18, 48, 66), and introduction of dominant negative Ras into endothelial cells inhibits VEGF-induced endothelial cell DNA synthesis, migration, and tube formation (48). Moreover, Ras signaling is sufficient to promote an angiogenic phenotype in primary endothelial cells in culture (49), suggesting that regulation of endothelial Ras signaling is essential for blood vessel formation. However, the mechanism by which VEGF regulates Ras and the precise role of Ras activation in mediating VEGF function in endothelial cells are presently not well understood.

Genetic experiments *in vivo* are consistent with a role for Ras in vascular development. Genetic ablation of *Son of Sevenless* (*SOS*) RasGEF, neurofibromatosis 1 (*NF1*) RasGAP, or *RasGAP* results in cardiovascular defects (7, 36, 76). Partial functional overlap exists within the *ras* family itself, as mice

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deficient for *N-ras*, *H-ras*, or both are normal (27, 74). *K-ras* has a unique and required function, as mice deficient for *K-ras* die progressively during development due to multiple defects, including defects in the hematopoietic and cardiovascular systems (40, 45). Although expression of Ras and these Ras regulators is not restricted to blood vessels, targeted deletion of *NF1* RasGAP in endothelial cells leads to multiple cardiovascular defects (34). This result indicates that NF1 is a required gene product in endothelial cells during blood vessel development and suggests that regulation of Ras signaling is important in angiogenesis *in vivo*. However, the role of Ras activation in blood vessel formation remains poorly understood.

We identified Ras guanine-releasing protein 3 (RasGRP3) as an endothelial cell-expressed GEF in an embryonic stem (ES) cell-based gene trap screen for genes expressed in blood vessels (37, 67). RasGRP3 is one of four members of the RasGRP family of RasGEFs that couple diacylglycerol (DAG) signaling to Ras activation (12, 22, 41, 46, 47, 55, 56, 73, 78, 81). While the different RasGRP proteins share similar mechanisms of regulation, they exhibit distinct patterns of tissue expression and specificity for Ras and Rap GTPases. We determined that vascular expression of RasGRP3 was restricted to sites of active blood vessel formation developmentally and in the adult. The *Rasgrp3* gene trap locus (*Rasgrp3^{gt}*) is a loss-of-function allele, yet homozygous *Rasgrp3^{gt/gt}* mice and embryos displayed no obvious vascular defects. However, wild-type ES cell-derived vessels responded to the phorbol ester phorbol myristate acetate (PMA), which mimics DAG activity, with extensive aberrant morphogenesis. These changes required RasGRP3 function, since *Rasgrp3^{gt/gt}* vessels were completely refractory to the phorbol ester-mediated effects. The aberrant morphogenesis induced by phorbol ester in wild-type vessels closely resembled the phenotype of vessels with up-regulated VEGF signaling, and we showed that RasGRP3 expression is up-regulated by VEGF stimulation of endothelial cells. Taken together, these results show that RasGRP3 is a VEGF-responsive vascular GEF that is essential for the endothelial response to phorbol esters. RasGRP3 functions in a pathway that is redundant developmentally, but its stimulation perturbs normal angiogenesis, suggesting that it may exacerbate DAG-mediated vascular phenotypes in diseases such as diabetes.

MATERIALS AND METHODS

Generation of *Rasgrp3^{+/-gt}* ES cells. ES cells were maintained and differentiated as previously reported (4, 42, 43). ES cells with a gene trap vector in the *Rasgrp3* locus (*Rasgrp3^{+/-gt}*) were identified by analysis of β -galactosidase (β -Gal) expression upon differentiation as previously described (37, 67). Briefly, the PT1-ATG vector, which contains a promoterless *lacZ* gene with its own start codon, was introduced into ES cells by electroporation, and selection was performed with G418. Drug-resistant colonies were picked, expanded, differentiated, and analyzed for β -Gal expression in blood vessels.

β -Gal detection. Differentiated ES cell cultures, embryos, and adult tissues were processed for β -Gal detection as previously described (42). Briefly, ES cell cultures were differentiated to day 6 to 8 and then fixed in glutaraldehyde fixative (0.2% glutaraldehyde, 5 mM EGTA [pH 7.3], 2 mM MgCl₂ in 0.1 M phosphate buffer [pH 7.3]) for 5 min at room temperature. After three washes with phosphate buffer, cultures were incubated overnight at 37°C in 5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside (X-Gal; Sigma) staining solution (0.625 mg of X-Gal/ml, 5 mM potassium ferrocyanide and 5 mM potassium ferricyanide in ES cell wash buffer). ES cell wash buffer consisted of 2 mM MgCl₂ and 0.02% NP-40 in 0.1 M sodium phosphate buffer (pH 7.3). After incubation, cultures were rinsed and stored in wash buffer at 4°C.

Embryos and adult tissues were rinsed in 1 \times phosphate-buffered saline (PBS)

and fixed in either glutaraldehyde fixative or 4% paraformaldehyde (PFA)-PBS for 20 min at room temperature. After two 10-min washes in embryo wash buffer (0.1 M phosphate buffer [pH 7.3], 0.1% sodium deoxycholate, 0.02% NP-40, 0.05% bovine serum albumin [Sigma]), tissues were incubated overnight at 37°C in X-Gal staining solution (wash buffer containing 1 mg of X-Gal/ml [Sigma], 5 mM ferrocyanide, and 5 mM ferricyanide). Tissues were then postfixed in the appropriate fixative and stored at 4°C until embedded. Adult tissues were frequently dissected into smaller pieces prior to fixation for better penetration.

RACE cloning. Rapid amplification of cDNA ends (RACE) cloning was done as previously described (67). Briefly, RNA was prepared from differentiated ES cell cultures with Trizol (Roche Applied Sciences, Indianapolis, Ind.) per the manufacturer's protocol. RACE cloning was performed with a 5' RACE kit (GIBCO/BRL, Grand Island, N.Y.) according to the manufacturer's instructions. RACE products were subcloned into the pCRII vector (Invitrogen, Carlsbad, Calif.).

Nucleic acid isolation and analysis. For Southern blot analysis, genomic DNA was digested with EcoRI, separated on a 0.8% agarose gel, and transferred to a nylon membrane. Hybridization was performed with a RasGRP3-specific cDNA probe generated by RACE cloning. This probe encompassed exons 2 to 4 of RasGRP3 and part of exon 1. Primer sequences for PCR genotyping were 5' ATC TGG AGC CCA TTG AGT TG 3' and 5' CAG GCC AGA ACC AAG AGG TC 3', flanking exon 3 of RasGRP3, and 5' TGC CCT TTC TCC TCC ATG AC 3' and 5' GCG GGC CTC TTC GCT ATT AC 3', which are specific for the gene trap (see Fig. 2A).

Total RNA was isolated from cells or tissues with Trizol Reagent, per the manufacturer's protocol, for Northern blot analysis. RNA was separated on a 1.2% formaldehyde-agarose gel and transferred to nitrocellulose. The membrane was hybridized in Quickhyb hybridization buffer (Stratagene, La Jolla, Calif.) with [³²P]dCTP-labeled cDNAs and then exposed to film. All radioactive probes were generated by random priming with the Prime-It RmT Random Primer labeling kit (Stratagene). Template for the mouse RasGRP3 Northern probe was generated using the primers 5' GCT CGG GAA AGC GGC AAC AC 3' and 5' ATT GGC GGT CTG GCT TTG G '3, corresponding to nucleotides 344 to 1575 of mouse RasGRP3. Human RasGRP3 probe was generated using a 772-bp HindIII fragment from a human RasGRP3 cDNA clone, KIAA0846 (51). This probe corresponds to nucleotides 2643 to 3415 of KIAA0846. The platelet endothelial cell adhesion molecule (PECAM) probe was generated from a 660-bp reverse transcriptase PCR (RT-PCR) product that corresponds to nucleotides 1244 to 1904 of PECAM.

Generation, maintenance, and genotyping of mice. Chimeric mice were produced from *Rasgrp3^{+/-gt}* ES cells by injection of ES cells into the blastocoel cavity of mouse blastocysts (32). The *Rasgrp3^{gt}* strain was established using three independent chimeras that demonstrated germ line transmission when bred to wild-type CD1 mice. For timed pregnancies, day 0.5 of gestation (E0.5) was noon of the day of vaginal plug observation. Genotyping was performed using DNA isolated from tail biopsies or embryonic yolk sac by Southern blotting or PCR analysis. MMTV-PyVT mice (the gift of W. Muller and T. Van Dyke) were crossed to *Rasgrp3^{gt}* mice through two rounds of matings to obtain MMTV-PyVT;*Rasgrp3^{+/-gt}* and MMTV-PyVT;*Rasgrp3^{gt/gt}* mice.

In situ hybridization. In situ hybridization on whole-mount embryos was performed by a modified protocol (61). The template for the RasGRP3 probe was the same template used for mouse Northern blot analysis. Briefly, mid-gestation embryos were isolated and fixed in 4% PFA-PBS overnight. Embryos were then dehydrated in a methanol-PBS series, followed by rehydration in a methanol-PBT (PBS with 0.1% Tween) series. Embryos were bleached in 6% H₂O₂ for 1 h, treated with 10 μ g of proteinase K/ml in PBT for 15 min, and then washed with fresh 2 mg of glycine in PBT/ml. Refixation was performed using 0.2% glutaraldehyde-4% PFA in PBT for 20 min. Embryos were incubated in prehybridization solution (50% formamide, 5 \times SSC [1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate] [pH 4.5], 0.1% Tween 20, and 0.05 mg of heparin/ml) for 1 h at 70°C prior to adding heat-shocked digoxigenin-labeled probe (1 μ g/ml). Hybridization was performed overnight at 70°C.

Following hybridization, embryos were washed according to protocol and then incubated with 100 μ g/ml RNase A for 30 min at 37°C. Blocking was performed using 10% lamb serum in Tris-buffered saline plus 0.1% Tween 20 (TBST) for 2.5 h at room temperature, and embryos were incubated with an alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim, Indianapolis, Ind.) at a 1:2,000 dilution overnight at 4°C. After antibody incubation, embryos were washed in TBST, and detection was with 50 mg of nitroblue toluene/ml and 30 mg of 5'-bromo-4-chloro-3-indolyl phosphate (BCIP)/ml (both from Promega, Milwaukee, Wis.) in 100 mM NaCl, 100 mM Tris-HCl (pH 9.5), 50 mM MgCl₂, 0.1% Tween 20, and 2 mM levamisole. When detection was

complete, embryos were washed in PBT (pH 5.5) and then postfixed in 4% PFA. Embryos were stored in PBS at 4°C.

RNA in situ hybridization of differentiated ES cell cultures was performed as previously reported (5) and as described above for embryos except for the following: day 8 cultures were fixed in 4% PFA for 1 h and dehydrated through a methanol-PBS series. Wells were rehydrated and then incubated in 6% H₂O₂ in PBT for 30 min, in proteinase K (3 µg/ml in PBT) for 10 min, and then in fresh glycine (2 mg/ml in PBT) for 4 min. Following the PBT washes, wells were refixed in 0.2% glutaraldehyde and 4% PFA in PBT for 8 min. Wells were hybridized as above except that an initial 3-h incubation was performed at 80°C prior to addition of probe. RNase treatment, blocking incubation, antibody reaction, and substrate development were all as previously described.

Antibody staining. Antibody staining of ES cell cultures was as previously described (5, 42). Rat anti-mouse PECAM was used at a 1:1,000 dilution (Mec 13.3; BD Pharmingen, San Diego, Calif.), and donkey anti-rat tetramethyl-rhodamine isothiocyanate (Jackson ImmunoResearch, West Grove, Pa.) was used at a 1:200 dilution. Cultures were stored at 4°C in PBS. Antibody staining of mid-gestation embryos was also as previously described (1, 2). Embryos were incubated with rat anti-mouse PECAM (Mec 13.3; BD Pharmingen) at a 1:200 dilution, followed by incubation with horseradish peroxidase-conjugated goat anti-rat secondary antibody (Pierce, Rockford, Ill.) at a 1:200 dilution. Detection was with 0.5 mg of 3',3'-diaminobenzidine tetrahydrochloride (Sigma)/ml, 3 mg of nickel sulfate/ml, and 0.03% hydrogen peroxide in PBS.

HUVEC stimulation. Human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, Md.) and maintained in endothelial growth medium 2 supplemented with the CC-4176 bullet kit per the manufacturer's protocol. HUVECs were utilized between passages 4 and 8. For RasGRP3 regulation experiments, HUVECs were grown to near-confluency, then serum starved overnight in M199 supplemented with 2% fetal bovine serum. HUVECs were then stimulated with various concentrations of VEGF or basic fibroblast growth factor (bFGF) for 24 h, and isolated RNA was analyzed on Northern blots. Recombinant human VEGF164 (R&D Systems) was used at 5 to 60 ng/ml, and recombinant human bFGF (Sigma) was used at 5 to 80 ng/ml.

Production of *Rasgrp3^{3gt/3gt}* ES cells. *Rasgrp3^{3gt/3gt}* ES cells were seeded at 5 × 10⁴ cells per 10-cm plate in ES cell medium (4, 43), and 1 day later, 640 µg of G418/ml was added to the medium. After 10 to 14 days of G418 selection, colonies were picked, expanded, and screened for homozygosity at the *Rasgrp3* locus by PCR and Southern blot analysis. Three different *Rasgrp3^{3gt/3gt}* ES cell clones were characterized.

PMA treatment of differentiating ES cell cultures. PMA (Calbiochem, San Diego, Calif.) was resuspended in dimethyl sulfoxide at 10 mM. PMA (10 to 1,000 nM) was added to differentiating ES cell cultures starting on day 5 of differentiation. Fresh PMA was then added daily. At day 8, cultures were fixed and processed for antibody staining as previously described.

RESULTS

Identification of a gene trap insertion in the *Rasgrp3* locus.

We carried out a gene trap screen in ES cells to identify novel genes expressed in developing blood vessels or blood cells (37, 67). The approach was to induce random insertion into ES cells of a vector that both lacked a promoter/enhancer and contained a splice acceptor site. Thus, expression of the reporter should be facilitated by insertion into an intron, and the expression pattern is predicted to reflect the expression pattern of the insertion locus. ES cells can be induced to undergo a programmed differentiation in vitro that recapitulates developmental processes and results in the formation of multiple tissues, including primitive blood vessels (4). The combination of gene trap insertion and ES cell differentiation to primitive vessels is a powerful approach for the identification of new genes and/or genes not previously associated with blood vessel formation.

One such gene trap ES cell line selectively expressed the β-Gal reporter gene in developing ES cell-derived blood vessels (Fig. 1A). Initial RACE cloning combined with analysis of expressed sequence tag databases and the mouse genome se-

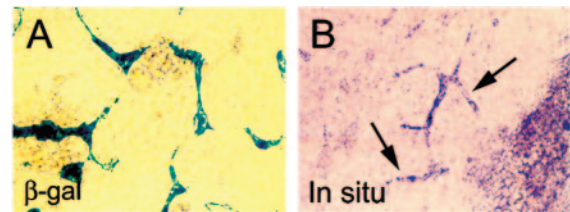


FIG. 1. RasGRP3 is expressed in ES-cell derived blood vessels. (A) ES cells with a *lacZ*-containing gene trap integration in the *Rasgrp3* locus (*Rasgrp3^{3gt/3gt}*) were differentiated to day 8 (day 8 ES cell cultures) and stained for β-Gal. β-Gal expression was detected in ES cell-derived blood vessels. (B) Wild-type (*Rasgrp3^{+/+}*) day 8 ES cell cultures were hybridized with an in situ hybridization probe specific for RasGRP3. Arrows point to several blood vessels that reacted with the probe.

quence indicated that the gene trap insertion occurred at the 5' end of a gene encoding the small GTPase activator RasGRP3 (Fig. 2A). To confirm that the insertion occurred in this locus, we performed Southern blot analysis of DNA from ES cells that were wild type (*Rasgrp3^{+/+}*), heterozygous (*Rasgrp3^{+/3gt}*), or homozygous (*Rasgrp3^{3gt/3gt}*) for the gene trap insertion (Fig. 2B). A band shift was detected in *Rasgrp3^{+/3gt}* and *Rasgrp3^{3gt/3gt}* DNA with a RasGRP3 probe, confirming that the gene trap insertion did indeed occur in the *Rasgrp3* locus.

Further analysis of the genomic DNA showed three hybridizing EcoRI fragments in wild-type DNA, but only two such bands in *Rasgrp3^{3gt/3gt}* DNA (Fig. 2B). Although other possibilities exist, the simplest explanation for this result is that the gene trap insertion deleted one of the *Rasgrp3* exons. To test this hypothesis, we performed PCR experiments to assay for the presence of exons 2, 3, and 4 in genomic DNA (Fig. 2C). All three primer sets amplified fragments from wild-type and heterozygous DNA, and exon 2 and 4 primer sets amplified fragments from *Rasgrp3^{3gt/3gt}* DNA. However, a PCR product corresponding to exon 3 was not amplified from *Rasgrp3^{3gt/3gt}* DNA, suggesting that exon 3 was deleted by the gene trap insertion (Fig. 2A). This result is consistent with the fragment sizes detected by Southern blot analysis and suggests that the *Rasgrp3^{3gt}* locus is a null allele, since exon 3 contains the AUG start codon of RasGRP3.

Analysis of the mouse genome sequence predicted that the mouse RasGRP3 transcript was approximately 4.2 kb and contained 18 exons. We cloned the predicted open reading frame of this transcript by RT-PCR from the mouse endothelial cell line Py-4-1 (21), and sequenced the transcript (Fig. 3A). The mouse RasGRP3 protein is predicted to be 691 amino acids, with four conserved domains shared by all RasGRP proteins: a REM (Ras exchange motif) domain, a CDC25 homology domain, a pair of atypical EF hands, and a regulatory C1 domain (Fig. 3B). The RasGRPs activate Ras and other small GTPases via the CDC25 homology catalytic domain, and they are activated in turn by the formation of DAG, which resides in the membrane. The C1 domain interacts with DAG, causing recruitment of RasGRP to the plasma membrane, where membrane-bound Ras is stimulated (Fig. 3C) (for a review, see reference 17). Phospholipase C-γ (PLC-γ) generates DAG by cleaving PIP₂, and PLC-γ is downstream of receptor tyrosine kinases such as the VEGF receptor (69). Mouse RasGRP3

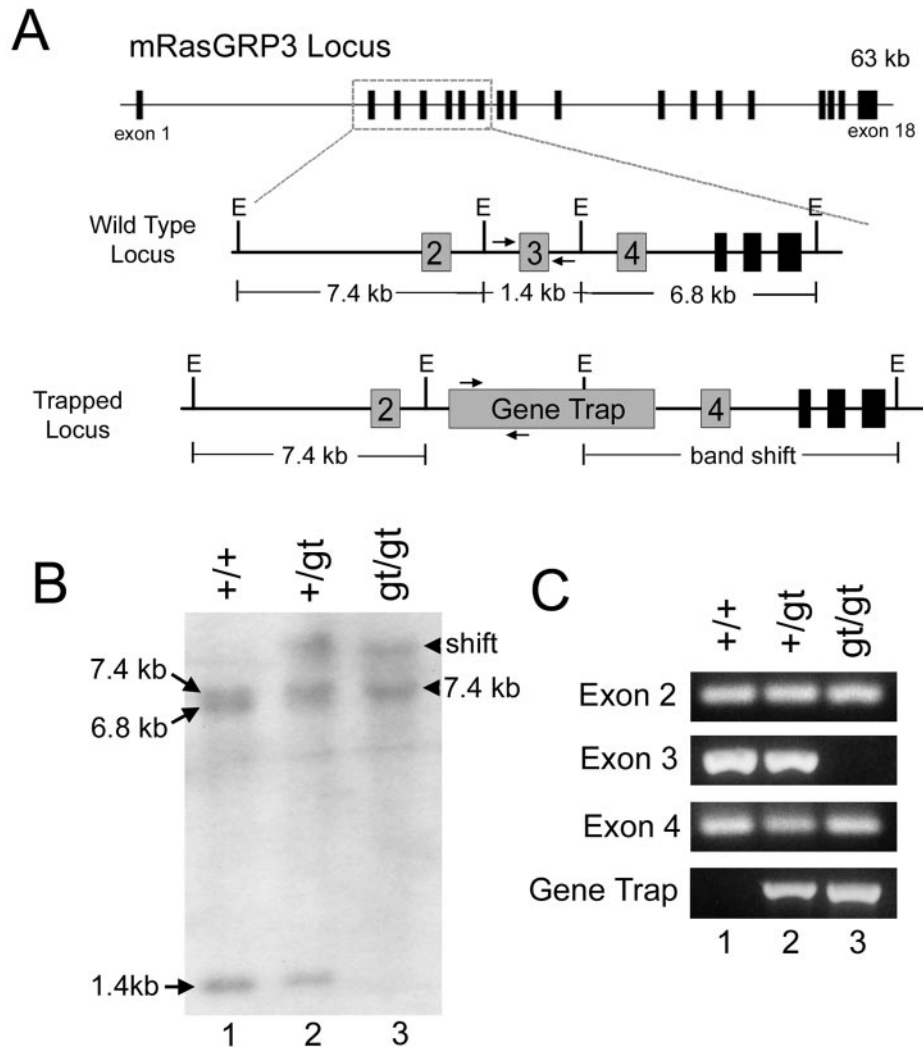


FIG. 2. The gene trap construct integrated into the *Rasgrp3* locus. (A) Schematic of the mouse *Rasgrp3* locus. The locus is approximately 63 kb and consists of 18 exons. The region encompassing exons 2 to 7 is enlarged to show the restriction fragments analyzed by Southern blot analysis. E, EcoRI sites. Arrows denote PCR primers for genotyping *Rasgrp3* mice. (B) Southern blot analysis of EcoRI-digested genomic DNA from *Rasgrp3*^{+/+} (lane 1), *Rasgrp3*^{+/gt} (lane 2), and *Rasgrp3*^{gt/gt} (lane 3) mice. The blot was hybridized with a cDNA probe consisting of exons 2 to 4. Arrows denote hybridizing fragments in *Rasgrp3*^{+/+} DNA. Arrowheads denote the hybridizing fragments in *Rasgrp3*^{+/gt} DNA. (C) Genomic PCR with primers specific for exons 2 to 4 and the gene trap. *Rasgrp3*^{+/+} (lane 1), *Rasgrp3*^{+/gt} (lane 2), and *Rasgrp3*^{gt/gt} (lane 3) are shown.

shares extensive nucleotide and protein homology to human RasGRP3 and to the other three RasGRP family members from both species (data not shown).

The expression of RasGRP3 in ES cell-derived blood vessels suggested that this activator might be involved in angiogenesis. Three criteria were considered important in assigning RasGRP3 a role in vascular processes: (i) vascular expression during embryonic development, (ii) endothelial cell expression during neoangiogenesis in the adult, and (iii) altered blood vessel formation upon perturbation of RasGRP3 function. These criteria were assessed with molecular, genetic, and pharmacological tools.

RasGRP3 is expressed in blood vessels during mouse development. Because Ras is a critical transducer of numerous signals important for cell proliferation and differentiation, we asked if RasGRP3 was expressed developmentally. To do this,

we generated *Rasgrp3*^{+/gt} mice from *Rasgrp3*^{+/gt} ES cells using standard techniques, and analyzed expression of RasGRP3 via the β -Gal reporter gene. We focused on a developmental window from E7.5 to E10.5 because this is when vascular development is most prominent. During development, β -Gal expression from the *Rasgrp3* locus was predominately found in blood vessels. In E9.5 and E10.5 embryos, β -Gal was strongly expressed in the developing intersomitic vessels and the dorsal aorta (Fig. 4A and B). The majority of head vessels and small vessels along the sides of the embryo also faintly expressed β -Gal. β -Gal expression in developing vessels was not uniform at a given stage, suggesting that RasGRP3 expression is modulated developmentally. β -Gal from the *Rasgrp3* locus was also expressed in a limited number of nonvascular embryonic cells such as the pericardium and the myotome of the somite (Fig. 4A and B and data not shown). Examination of transverse

A

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1 catgcgccacctggcagccagacagggccaagcgctgggtgaggaagacaaaattaccgccagtttccagagcccttatcatttgagtttaacgaaccagcttc
106 ttcactctgttccctcatgcccctcccagggatgacaactggccctgggacatcttccagacctggnaactctctctgtagaasaatagatcattcttgaagt
211 ataacttaagaaagaaactacctgccaagggagagaaacactgcccagagataggatagctctctgctgctggaaatgctccagatcttgaaca
1 M G S N G L G K A A T L D E L L S T C I E M F D D N G D L N D
316 acggtaasgaaccATGGGGTCCAACGGGCTCGGGAAGCGCAACACTAGATGAACCTTCAGCACATGCATGAGATGTTGATGCAACTGGAGACCTGAAGC
32 SYLPRIIVLLMHRWYLSSTELAGKLLCYRNAASGES
421 ACAGTTACTGCCAGAACTGGTTACTGATGCACCGATGGTATTATCTCCACTGAACGGCAGAAACCTCCTCGCATGTATCGGAATGCCAGTGGAGAA
67 C D E F R E K I C Y F M R Y N I L K F P A E F N L D L G L T R M T F E
526 GCCTGTGATGAGTCCGACTGAAGATCTGCTATTCTAGGAGTCTGGATTCTGAAATTCCTGCAGAGTTAATTTGGATCTGGTGTATCCGATGACTGAGG
102 F R E V A S O L G H E K H I S L M D I S S V P S Y D W M R R V T Q R K
31 AGTTCGGGAGTAGCTAGTCACTAGGACATGAAACACATCAGCCTCATGGACATATCTAGCGTCTCTCTATGACTGGAAGAGGAGCTCACACAGAGGA
137 K V S K R G K A C L L F D H L E P I E L A E H L T F L E H K S F R R I
736 AAAAAGTATCCAAAAGGGGAAAGCCCTGCTCTTATTGACCATCTGGAGCCATTAAGTTCGGGAGCACCCTCCTTTCTGGAGCATAAATCTTTAGAGGA
172 S F T D Y Q S Y V I H G C L E N N P T L E R S I A L F N G I S K W V Q
841 TCTCATCCAGATATCAGAGCTATGTCATCCATGGCTGCTGGAGAATAACCCGACATGGAGAGATCTATGCTTATTAATGAACTCTAAAGTGGCTC
207 L M V L S K P S A Q Q R A E V I T K F I N V A Q K L L Q L K N F N T L
946 AGTTGATGGTCTTAGCAAAACCAAGTGGCACAAGAGGGCAGAAGTCTACAAAGTTCACTCAATGTAGCCAGAAACCTCTCAACTAAAATTTTACACCT
242 H A V V G G L S H S S T S R L K D T H S H L S S F V T K K W N E M T E
1051 TGATGGCAGTGGTGGTGGCTTAGTCAACAGCTCAATTCGCCACTCAAAGACACCCATTCACCTCTCTCCGAAGTACAAGAACCTGGATGAAATGAGACG
277 L V S S N G N Y C N Y R K A F A D C D G F K I P I L G V H L K D L I A
1156 AGTTGGTCTCTCAATGGCAATFACTGCAATTACCGCAAGCCTTTGCTGACTGTGATGGCTCAAAATCCCATCCTTGGGGTGCACCTGAAAGACTGTATG
312 V H V T F P D W M E E N R V N V K H Q L S V T L E S L V S L Q N A
1261 CAGTCCATGTCAATTTCCAGACTGGATGGAGGAGACAAGTGAACGTTTGAAATGACACCTTTCTGTTACCTGAGTGAACCTGGCTCCCTCGAGAATG
347 S H H L E P N M D L I N L L T L S L D L V H T E D D I Y K L S L V L E
1366 CCTCTACCACCTGGAACTAACATGGATTGATCAACTACTACTCTCTCTGGACCTTACCACACAGAGGATGATATAACAACTGCTCACTGGTGTGG
382 P R N S K S Q P T S P T T P N K P V V P L E W A S E V V P K P D P T I
1471 AGCCTAGAAATTCCAATCGCAGCAACTCCCAACGACACTAATAAGCCTGTGTACTCTGGAGTGGGCTTCAGAGTGGTACCAAGCCAGACCCACAA
417 I N K H I R K L V E S V F R N Y D H D H D G Y I S Q F D F E S I A A N
1576 TCATCAACAACACATCAAGGAAGTGGTGGTCTGTGTTTCAAACTACGATATGACCCAGATGGTACATCTCCCAAGAGGACTTGGAGAGTACCTGCCA
452 F P F L D S F C V L D K D Q D G L I S K D E M M A Y F L R A K S Q L H
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487 C R M G P G F I H N F Q E M N Y L K P T F C E H C A G F L W G I I K Q
1786 ACTCAAAATGGACAGGCTTCACTCAATTTTCAGAGAGTGAACCTTCAAGCCACTCTCCGAGACTGCGGGATTTCTGGGGCAATTAATCAAC
522 G Y K C K D C G A N C H K Q C K D L L V L A C R R L A R A P S L S S N
1891 AAGGCTACAAGTGAAGACTGTGGAGCCACTGCCAAGCAGTGAAGACCTTGGTCTGGCTCGAGGAGACTTGCAGGGCACTCTTGGAGCAGCA
1857 P G S L P G S P A L P P V Q D E V F E F P G V T A G H R D L D S R A I
1996 ACCCGGGTCACTGCCTGGAGCCAGCCTTGCCTCCAGTGCAGGATTTGAGTTCTGGGGTCAAGGCTGGACACCCAGACTGGACAGCAGCAAGCA
592 T L V T G S R K T S V R L Q R A T S G A T Q T E P V W S E V W G
2101 TTACTCGTGTACAGGATCTCCCGAAGATCTCCGTGAGCTGCAGAGCCACCCTAGTCAAGCCACCCAGAGCACTGTGTGGTCCAGGGCTGTCTGGG
627 D S G S H T F P K M K S K F H D K A A K D K G F A K W E N E K P T V Q
2206 GAGACTCAGGTTCCATACCTTCCCAAAATGAATCAAGTTCATGACAAGCAGCAAGGACAGGGCTTCCCAAGTGGGAAACGAGAGCCACAGGATC
662 A G V D V D R G T A F E P D Q D D G G D E A K Q G E D G
2311 AAGCTGTGGATGTGTAGATGAGGACTCCTATTGAACTGACAGGATGATGGCAAGATGAAGCAAGCAGGTTGGTGGATGAGTCAAGTCAAGTCAAGT
2416 gggaaacaaagcaactcaagtcggatcagaggaggagagaaaggcgctctgaaagggcaggtctctcagaagcggtctggaaagagagattgggatatgc
2521 tcaagcctccagtcactcggatccccactgactggaactcagggatagagaattaccctgactgaaactatttattctctgactctgctgcccctgctgagg
2626 tggcagaaactactaaagcctccccactcctaaagccatttgtagccagggggatgaaagcttgggtgcaagtcggtggtttctctcaaaatcaaaa
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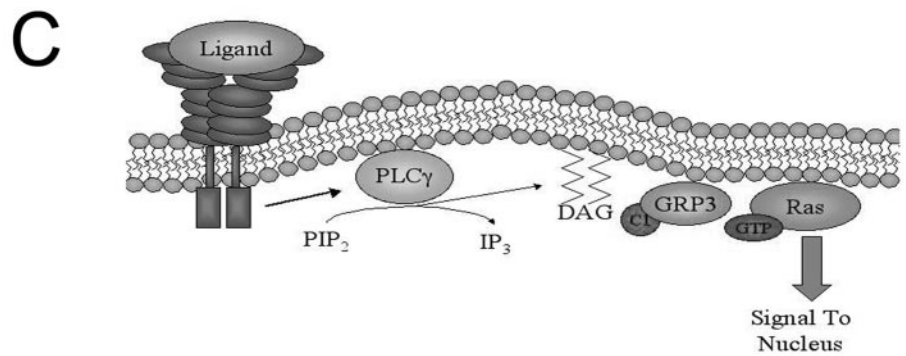


FIG. 3. Cloning and analysis of the RasGRP3 cDNA. (A) Full-length RasGRP3 was cloned from the mouse endothelial cell line Py-4-1 by RT-PCR and sequenced. Underlined sequences represent conserved domains. (B) Schematic of RasGRP3 protein. REM, RasGEF N-terminal domain; CDC25, catalytic RasGEF domain; EF, atypical EF hand domain; C1, PKC region 1 domain that binds DAG. (C) Model of RasGRP3 activation and signaling. Upon ligand-receptor engagement, PLC- γ is activated, which leads to the production of DAG from PIP $_2$. DAG is localized to the plasma membrane. C1 domain binding of DAG recruits RasGRP3 to the membrane where Ras activation occurs.

sections at E9.5 revealed β -Gal expression in the dorsal aortae, cardinal veins, and the perineural vascular plexus that surrounds the developing spinal cord (Fig. 4C).

To determine if β -Gal expression from the *Rasgrp3* locus faithfully recapitulated expression of the endogenous gene, we utilized whole-mount in situ hybridization with a probe for RasGRP3 that did not recognize other family members. RasGRP3 RNA was detected in ES cell-derived vessels, the dorsal aorta, and intersomitic vessels and faintly in the small vessels of the head (Fig. 1B and 4D). This expression pattern closely

resembles the whole-mount β -Gal expression pattern, confirming that the β -Gal reporter recapitulates vascular expression of endogenous RasGRP3.

RasGRP3 expression is modulated during angiogenesis and by VEGF. Although RasGRP3 was expressed in blood vessels of the developing embryo, the majority of vessels in the adult mouse lacked RasGRP3 β -Gal expression (Fig. 5A and B). Additionally, there was only rare expression of RasGRP3 β -Gal in adult nonvascular cells in organs such as the brain and spleen (data not shown). Since RasGRP3 was expressed in

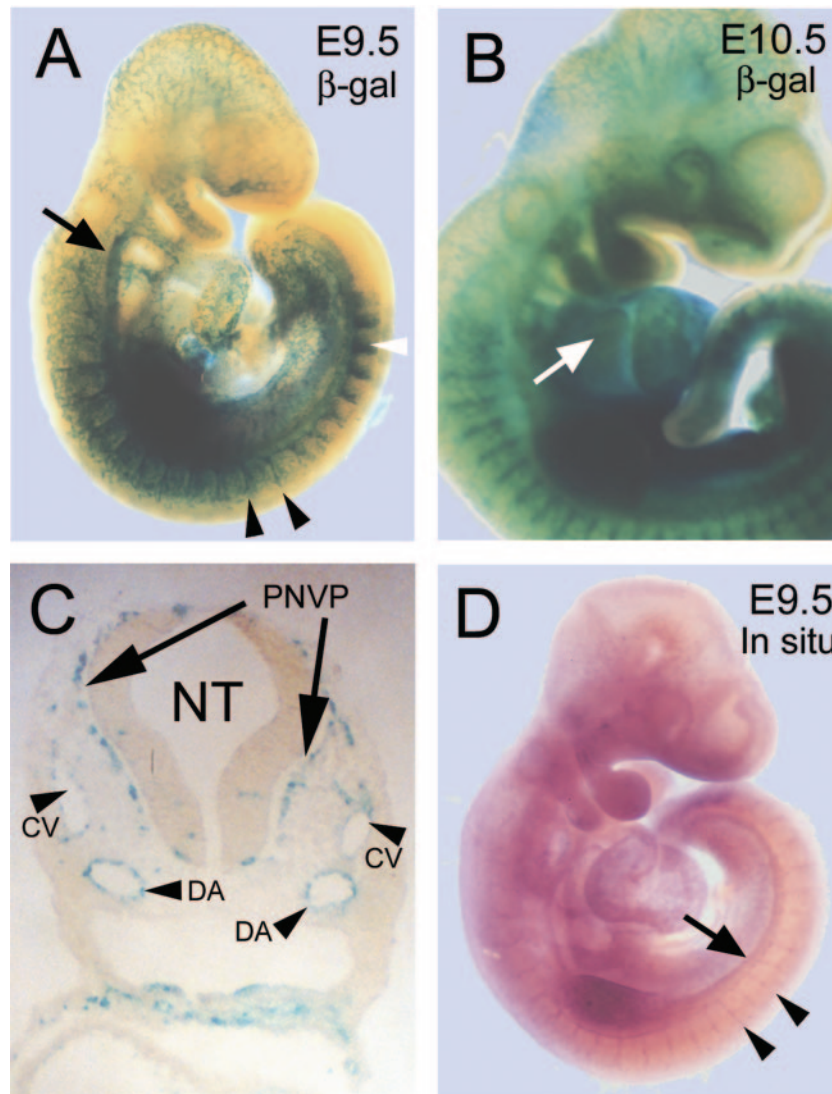


FIG. 4. RasGRP3 is expressed in blood vessels developmentally. (A and B) Embryos at E9.5 (A) and E10.5 (B) with *lacZ* inserted into the *Rasgrp3* locus (*Rasgrp3^{+/lacZ}*) were stained for β -Gal. β -Gal expression was detected in the dorsal aorta, the intersomitic vessels, and many of the smaller vessels throughout the embryo. The black arrow points to RasGRP3 β -Gal expression in the dorsal aorta, black arrowheads indicate expression in the intersomitic vessels, the white arrowhead indicates somitic myotome expression, and the white arrow indicates expression in the pericardium. (C) Transverse section of an E9.5 embryo stained for RasGRP3 β -Gal. NT, neural tube; PNVP, perineural vascular plexus; CV, cardinal veins; DA, dorsal aortae. (D) Whole-mount in situ hybridization of *Rasgrp3^{+/+}* wild-type E9.5 embryo for RasGRP3. RasGRP3 mRNA was detected in the dorsal aorta, intersomitic vessels, and several smaller vessels throughout the embryo. The arrow designates expression in the dorsal aorta, whereas arrowheads indicate expression in the intersomitic vessels.

embryonic vessels undergoing angiogenesis, we asked whether vascular RasGRP3 expression is up-regulated during adult angiogenesis. During pregnancy, vessels of the female reproductive tract undergo extensive expansion to provide nutrients and oxygen to the developing fetus. RasGRP3 β -Gal was expressed in maternal vessels of the decidua that surrounds the murine embryo, suggesting that RasGRP3 is reexpressed in these adult angiogenic vessels (Fig. 5C).

As tumors proliferate, they recruit host-derived blood vessels via angiogenesis. To address regulation of RasGRP3 in tumor vessels, we first took advantage of a *Rasgrp3^{3^{gt}/lacZ}* mouse that developed spontaneous sarcomas. We found that RasGRP3 β -Gal was reexpressed in these tumor blood vessels

(Fig. 5D). To further extend this analysis, we obtained MMTV-PyVT transgenic mice, and bred this transgene into the *Rasgrp3^{3^{gt}}* background. MMTV-PyVT mice rapidly develop mammary tumors as a consequence of PyVT oncogene expression in the mammary glands, and the resulting tumors are highly angiogenic (35). Analysis of MMTV-PyVT;*Rasgrp3^{3^{gt}/lacZ}* mice revealed that RasGRP3 β -Gal was also reexpressed in the vessels of these tumors (Fig. 5E). Taken together, these results indicate that vascular RasGRP3 expression is up-regulated during both normal and pathological angiogenesis.

Although most adult vessels did not express RasGRP3, RasGRP3 was expressed constitutively in the kidney glomerulus (Fig. 6A and B) as was previously reported (78). VEGF ex-

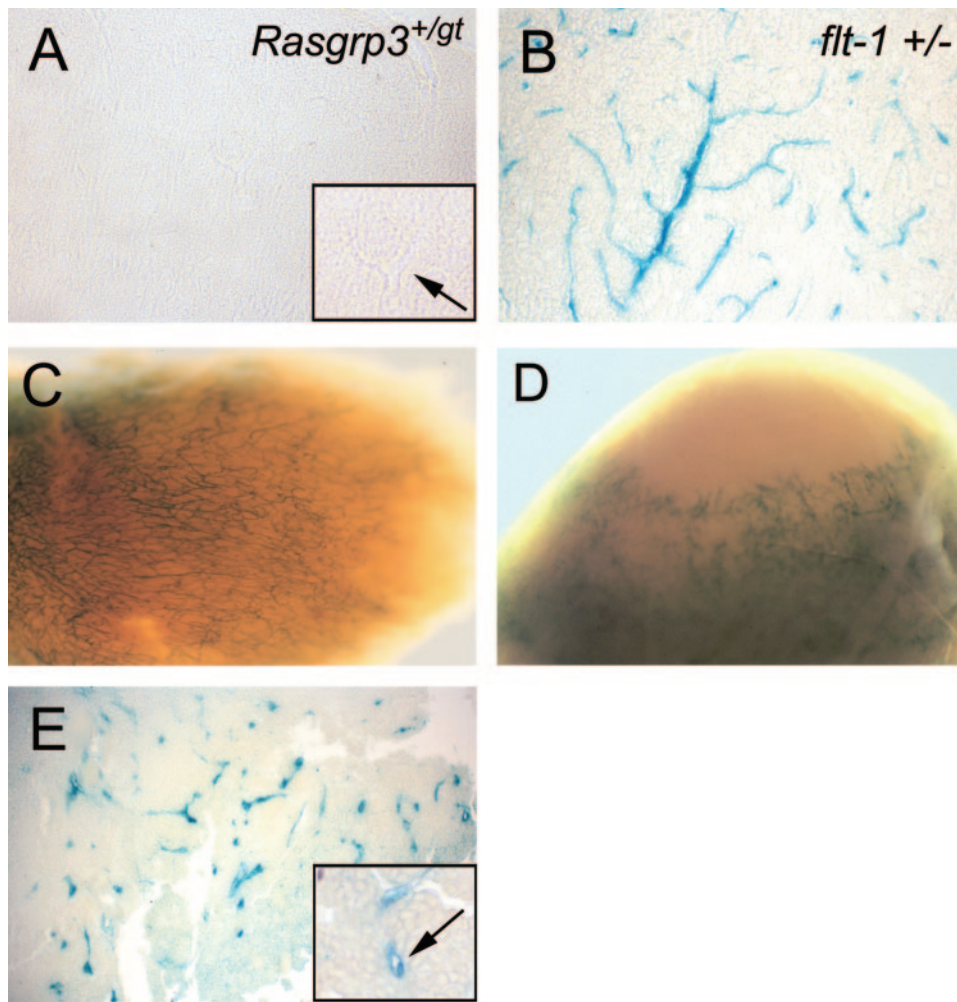


FIG. 5. RasGRP3 expression is up-regulated during normal and pathological adult angiogenesis. (A) Brain section from a *Rasgrp3*^{+/gt} mouse stained for β -Gal. Note the absence of β -Gal staining in the blood vessels. The insert shows a vessel (black arrow) at higher magnification that does not express β -Gal. (B) Equivalent brain section from a *flt-1*^{+/-} mouse with *lacZ* inserted in the *flt-1* locus. Note the β -Gal expression in the brain blood vessels. (C) *Rasgrp3*^{gt/gt} decidua stained for β -Gal. Note expression of β -Gal in decidual vessels. (D) Spontaneous tumor from a *Rasgrp3*^{gt/gt} mouse, stained for β -Gal. Note expression of β -Gal in tumor vessels. (E) Section of a mammary tumor from a MMTV-PyVT;*Rasgrp3*^{gt/gt} mouse stained for β -Gal. Note the expression of β -Gal in tumor vessels. The insert shows a β -Gal-positive vessel at a higher magnification. The arrow points to the lumenized vessel.

pression is also maintained in adult kidney glomeruli (9, 25); the VEGF receptor, flk-1 (VEGFR-2), is also expressed at this site (Fig. 6C and D) (58), indicating that VEGF signaling is intact in the glomerulus. Thus, VEGF expression is associated with neoangiogenesis in the embryo and the adult, and it is maintained in the glomerulus of the kidney, all sites of RasGRP3 expression. This coincidence suggested that VEGF signaling through flk-1 may induce RasGRP3 expression in vivo.

We tested this hypothesis by treating primary endothelial cells in culture (HUVECs) with increasing concentrations of recombinant VEGF. RasGRP3 RNA was barely detectable in serum-starved HUVECs by Northern blot analysis; however, VEGF stimulation resulted in up-regulation of RasGRP3 transcription in a dose-dependent manner, indicating that *Rasgrp3* is a VEGF-responsive gene (Fig. 7A). This effect was not universal to growth factor stimulation, as HUVEC stimulated

with another angiogenic growth factor, bFGF, did not show increased RasGRP3 RNA levels (Fig. 7B).

RasGRP3 is a phorbol ester receptor that functions in endothelial cells. The RasGRP3 gene trap insertion most likely deleted exon 3 of *Rasgrp3*, which contains the predicted start codon for the RasGRP3 protein, so the gene trap insertion is likely to have resulted in a null mutation (Fig. 2A). To further investigate this possibility, we analyzed total RNA isolated from *Rasgrp3*^{+/+}, *Rasgrp3*^{+/gt}, and *Rasgrp3*^{gt/gt} E9.5 embryos and day 8 ES cell cultures for RasGRP3 mRNA by Northern blot analysis (Fig. 7C). In all cases, RasGRP3 mRNA was readily detectable in *Rasgrp3*^{+/+} and *Rasgrp3*^{+/gt} samples; however, no RasGRP3 transcript could be detected in *Rasgrp3*^{gt/gt} samples. This result strongly indicates that the gene trap insertion resulted in a null mutation in *Rasgrp3*, although we cannot exclude the formal possibility that it is a severe hypo-

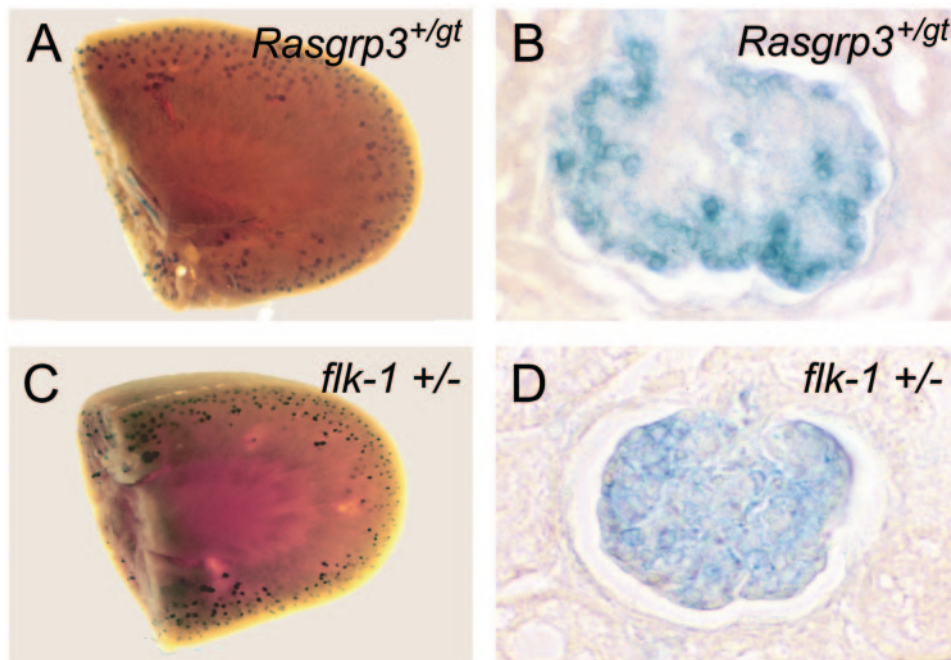


FIG. 6. RasGRP3 and flk-1 are both expressed in the glomerulus of the kidney. Kidneys from *Rasgrp3*^{+/*gt*} mice and *flk-1*^{+/*-*} mice with *lacZ* inserted into the *flk-1* locus were dissected and stained for β -Gal. (A and C) Whole-mount view of β -Gal expression in *Rasgrp3*^{+/*gt*} (A) and *flk-1*^{+/*-*} (C) kidneys. Note the peripheral, punctate expression of β -Gal in both backgrounds. (B and D) Kidney sections from *Rasgrp3*^{+/*gt*} (B) and *flk-1*^{+/*-*} (D) mice stained for β -Gal. Note that the β -Gal reporter is expressed in the glomerulus in both genetic backgrounds.

morphic mutation. Moreover, we were unable to detect expression of RasGRP3 in *Rasgrp3*^{gt/gt} embryos and day 8 ES cell cultures by in situ hybridization (data not shown), further supporting the conclusion that functional RasGRP3 RNA is not made from the *Rasgrp3*^{gt} locus.

To determine if loss of RasGRP3 affected development, we intercrossed *Rasgrp3*^{+/*gt*} mice and analyzed the progeny. *Rasgrp3*^{gt/gt} mice were born at the expected Mendelian ratio, and the life spans of these mice were comparable to those of littermate controls, indicating that RasGRP3 is not required for development or viability (data not shown). Furthermore, no differences in reproductive ability were observed. The blood vessels of *Rasgrp3*^{gt/gt} mice also appeared normal, as no hemorrhage or morphological defects were detected (data not shown). Because RasGRP3 expression is regulated developmentally, we asked whether *Rasgrp3*^{gt/gt} embryos had a subtle vascular phenotype that was rescued prior to birth. To address this issue, we isolated mid-gestation embryos from *Rasgrp3*^{+/*gt*} intercrosses and stained them with the vascular marker PECAM. Compared to blood vessels of littermate controls, the blood vessels of *Rasgrp3*^{gt/gt} embryos appeared normal (Fig. 8A to C). Consistent with these in vivo results, the vasculature of differentiated *Rasgrp3*^{gt/gt} ES cell cultures also appeared normal (Fig. 8D to F). The *Rasgrp3*^{gt/gt} vessels were the proper thickness and branched appropriately, with normal variation in those parameters as seen in wild-type controls.

Since RasGRP3 was up-regulated during tumor angiogenesis, the consequences of RasGRP3 deficiency during tumor development were investigated. Compared to littermate controls, the kinetics and frequency of mammary tumor development in MMTV-PyVT;*Rasgrp3*^{gt/gt} mice were not affected (data

not shown). We also examined the kidneys of *Rasgrp3*^{gt/gt} mice by histology and found no obvious lesions or abnormalities (data not shown). Taken together, these results indicate that RasGRP3 deficiency does not result in an obvious vascular phenotype at the stages and sites analyzed.

RasGRP3 localization and activity is regulated by DAG, and this interaction is mimicked by the phorbol ester PMA. Thus, PMA is predicted to increase RasGRP3 activity and Ras activation (47). Normally, ES cell-derived blood vessels form a vascular plexus consisting of thin vessels that branch at regular intervals (Fig. 9A to C); however, wild-type ES cell-derived vessels treated with PMA underwent extensive alterations and formed large sheets with little branching (Fig. 9D). Formation of these endothelial sheets required RasGRP3 activity, since *RasGRP3*^{gt/gt} cultures treated with PMA did not form vascular sheets, but instead had morphologically normal vessels (Fig. 9F). This was true of three independent isolates of homozygous *Rasgrp3*^{gt/gt} ES cells. *RasGRP3*^{+/*gt*} cultures treated with PMA displayed an intermediate phenotype (Fig. 9E), suggesting a dose response to the PMA-induced morphological changes mediated by RasGRP3. PMA-induced morphological changes in vessels occurred over a range of PMA concentrations from 10 to 1,000 nM (data not shown).

The alteration of vessels observed following PMA addition to wild-type ES cell cultures closely resembles the vascular phenotype resulting from genetic ablation of the VEGF receptor, flt-1 (Fig. 9G), which up-regulates VEGF signaling. Because the PMA-induced vascular changes required RasGRP3 activity, we asked whether RasGRP3 was up-regulated in the *flt-1*^{-/*-*} background. Northern blot analysis revealed that, when normalized to the amount of vasculature, RasGRP3

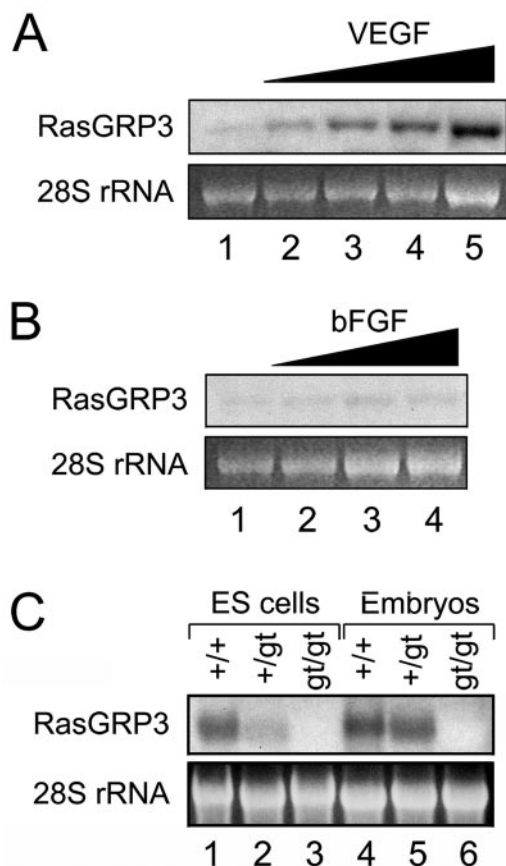


FIG. 7. *Rasgrp3* is a VEGF-responsive gene and a loss-of-function mutation. (A and B) HUVECs were incubated with increasing concentrations of VEGF (5 to 60 ng/ml) (A) or bFGF (5 to 80 ng/ml) (B). After 24 h, RNA was collected and subjected to Northern blot analysis. (C) RasGRP3 Northern blot analysis was performed on RNA isolated from *Rasgrp3*^{+/+}, *Rasgrp3*^{+/gt}, and *Rasgrp3*^{gt/gt} E9.5 embryos and day 8 ES cell cultures.

RNA levels were increased 1.5-fold in *flt-1*^{-/-} cultures (Fig. 10). Taken together, these results suggest that increased RasGRP3 activity may contribute to the aberrant morphology of *flt-1*^{-/-} vessels.

DISCUSSION

This study identifies RasGRP3 as a GEF expressed in the vasculature. Its expression is associated with developing vessels in the embryo and in the adult, suggesting that Ras activation through RasGRP3 is utilized for angiogenesis. Indeed, RasGRP3 expression is directly regulated by VEGF signaling, and the VEGF pathway is critical for angiogenesis. Moreover, RasGRP3 is required to mediate the aberrant endothelial morphogenesis that results from PMA exposure, a treatment that phenocopies aspects of up-regulated VEGF signaling. Taken together, our results indicate that RasGRP3 is a phorbol ester receptor that functions in endothelial cells, where it transduces signals that modulate angiogenic activity.

Endothelial RasGRP3 expression is regulated by angiogenesis and VEGF. RasGRP3 belongs to a GEF subfamily of four highly related proteins that contain a C1 domain. This domain

binds DAG, a membrane-localized second messenger, and brings the GEF into close proximity to membrane-localized GTPases to effect its activation function. Mouse RasGRP3 shares extensive sequence homology with human RasGRP3 and with the other family members (78). RasGRP family members, including RasGRP3, are expressed in hematopoietic cells: RasGRP1 is expressed in T cells, RasGRP4 is expressed in myeloid cells, and RasGRP3 is expressed in B cells (19, 23, 53, 56, 81). RasGRP2 has a more ubiquitous hematopoietic expression profile (G. Reuther and C. Der, personal communication), and RasGRP1 to RasGRP3 are expressed in some nonhematopoietic tissues such as brain and kidney (78). Given that endothelial cells share expression of numerous genes with hematopoietic cells, it is not surprising that we identified murine RasGRP3 in our gene trap screen to uncover novel vascular genes. B cells do not mature from ES cells in our differentiation protocol, and they are not present in mid-gestation embryos, so we did not score B-cell expression of RasGRP3. We did score expression of RasGRP3 using the β -Gal reporter readout in embryonic tissues such as the somitic myotome and the pericardium and in rare nonendothelial adult cells. However, RasGRP3 expression was most consistently found in blood vessels undergoing angiogenesis in both embryos and adults, suggesting that Ras activation through RasGRP3 accompanies angiogenesis.

The up-regulation of RasGRP3 expression during angiogenesis suggested that angiogenic signaling may regulate expression of this locus. VEGF signaling is a prominent feature of angiogenesis throughout life, and RasGRP3 expression is up-regulated upon VEGF stimulation of primary endothelial cells. VEGF-dependent up-regulation of RasGRP3 was also documented in a microarray analysis (80). Although VEGF signaling is dramatically down-regulated in quiescent vessels, the pathway remains active in the glomerulus of the kidney, where it is required to establish and maintain endothelial fenestrations for filtration (25, 26, 60). Likewise, RasGRP3 expression is maintained at this site, suggesting that Ras activation via RasGRP3 is involved in this process. RasGRP3 expression is also up-regulated in the maternal vessels of the decidua and in vessels that form in response to tumor angiogenic signals. These findings are consistent with a model in which RasGRP3 expression is downstream of VEGF signaling and VEGF stimulation increases the amount of RasGRP3 RNA in HUVECs. It will be interesting to determine the intracellular pathways downstream of VEGF that mediate up-regulation of RasGRP3 expression.

RasGRP3 is not necessary for angiogenesis but mediates the endothelial response to phorbol esters. Numerous studies implicate Ras signaling in endothelial function and vascular development (34, 36, 76). Generation of a *Rasgrp3* loss-of-function allele allowed us to assess the effects of RasGRP3 deficiency in developing vessels. Homozygous *Rasgrp3*^{gt/gt} ES cell-derived vessels formed normally, and *Rasgrp3*^{gt/gt} mutant embryos and mice had no obvious phenotype. RasGRP2 is required for proper platelet aggregation (13) and RasGRP1 is required for T-cell maturation in vivo (19), but we did not observe similar defects in *Rasgrp3*^{gt/gt} endothelial cells. This is not surprising, because redundancy for RasGRP3 function is predicted. It is possible that expression of other RasGRPs and/or other RasGEFs provides a compensatory activating

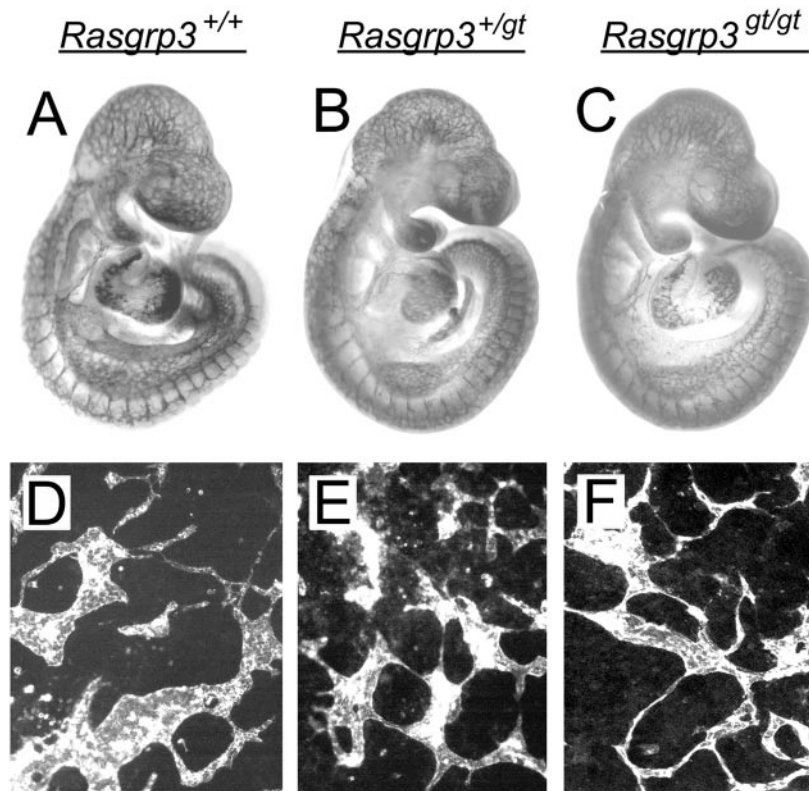


FIG. 8. *Rasgrp3*^{gt/gt} embryos and ES cell cultures do not have an obvious vascular phenotype. *Rasgrp3*^{+/+}, *Rasgrp3*^{+/gt}, and *Rasgrp3*^{gt/gt} E9.5 embryos and day 8 ES cell cultures were incubated with anti-PECAM antibody and reacted with 3',3'-diaminobenzidine tetrahydrochloride (A to C) or an immunofluorescent secondary antibody (D to F). *Rasgrp3*^{+/+} (A and D), *Rasgrp3*^{+/gt} (B and E), and *Rasgrp3*^{gt/gt} (C and F) embryos or cultures are shown. Note the lack of differences among the genotypes.

function. We detected RasGRP2 but not RasGRP1 or RasGRP4 in a murine endothelial cell line by RT-PCR analysis, suggesting that RasGRP2 function may compensate for loss of RasGRP3 (D. Roberts and V. Bautch, unpublished results). However, RasGRP2 is a Rap1 activator and not a Ras activa-

tor, whereas RasGRP3 is an activator of Ras and Rap1. Therefore, other RasGEFs may also compensate for the loss of RasGRP3 regulation of Ras. There are multiple RasGEFs expressed in most cells, and other GEFs such as the *Drosophila melanogaster* RhoGEF GEF64C do not exhibit a loss-of-func-

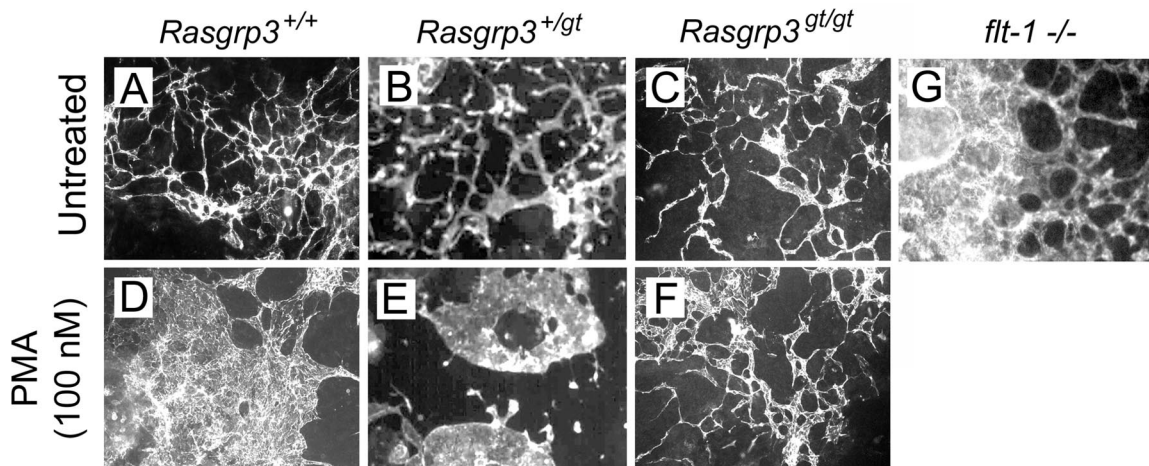


FIG. 9. PMA-induced morphological alteration of blood vessels requires RasGRP3 function. Day 5 to 8 ES cell cultures were treated with PMA, fixed on day 8, and reacted with anti-PECAM antibody. (A to C) Untreated; (D to F) 100 nM PMA. *Rasgrp3*^{+/+} (A and D), *Rasgrp3*^{+/gt} (B and E), *Rasgrp3*^{gt/gt} (C and F), and *flt-1*^{-/-} (G) cultures are shown. Note the formation of PECAM-positive endothelial sheets in the PMA-treated *Rasgrp3*^{+/+} cultures and the intermediate structures in *Rasgrp3*^{+/gt} cultures. Cultures lacking *flt-1* also formed endothelial sheets.

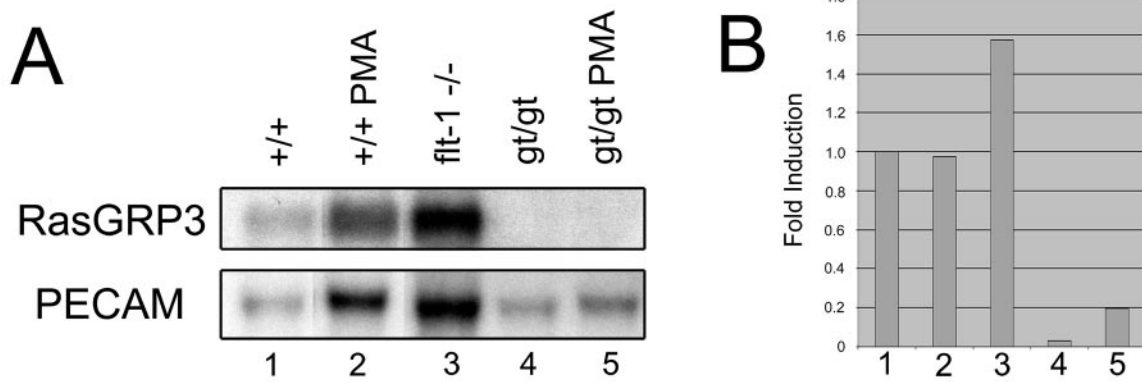


FIG. 10. RasGRP3 is upregulated in *flt-1*^{-/-} blood vessels. (A) Northern blot analysis was performed on RNA isolated from *flt-1*^{-/-} cultures (lane 3). RNA was also isolated from *Rasgrp3*^{+/+} and *Rasgrp3*^{st/st} cultures treated with PMA (lanes 2 and 5) or left untreated (lanes 1 and 4). The blot was probed for RasGRP3 and then reprobbed for the vascular marker PECAM. (B) Graph showing relative levels of RasGRP3 RNA in ES cell cultures. RasGRP3 RNA levels were normalized to the amount of PECAM RNA.

tion phenotype (3). One prominent RasGEF that is expressed ubiquitously is the mammalian Son-of-Sevenless (SOS) homologue (20). This GEF is complexed with the adaptor protein Grb2, and the SOS-Grb2 complex translocates to the plasma membrane via Grb2 association with the cytoplasmic domain of activated receptor tyrosine kinases, thus promoting SOS activation of Ras. Studies with B cells suggest that SOS and RasGRP3 may activate Ras coordinately (53); therefore, SOS may be able to partially compensate for the lack of RasGRP3 activity in endothelial cells (Fig. 11). Ras activation can also occur independently of RasGEFs. Protein kinase C (PKC) activation of extracellular signal-regulated kinases 1 and 2 in

endothelial cells is insensitive to Ras-N17, a dominant negative form of Ras that sequesters and inactivates RasGEFs (18, 66, 70). In this case, PKC is thought to activate sphingosine kinase (SPK), and SPK may activate Ras by RasGAP inhibition (Fig. 11). However, PKC also phosphorylates RasGRP3, and this phosphorylation event is highly correlated with membrane localization of RasGRP3 and Ras activation in B cells (8, 71). This finding suggests that PKC may affect cellular functions at least in part via activation of RasGRP3.

We thus asked whether a gain-of-function manipulation of RasGRP3 produced a vascular phenotype. Other GEFs up-regulate their target pathways when they are constitutively

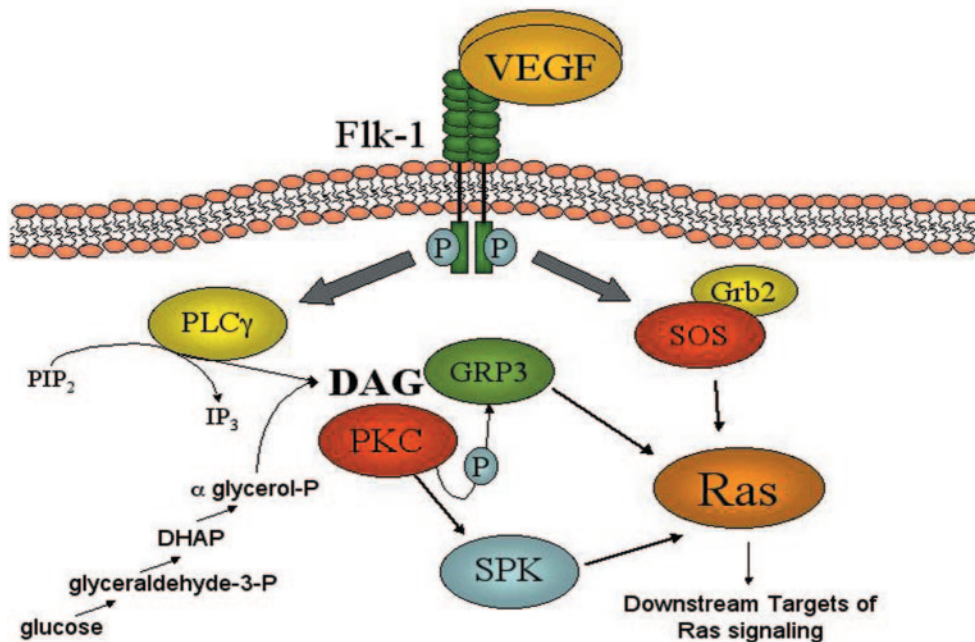


FIG. 11. Model of Ras activation downstream of VEGF signaling. Several potential pathways downstream of VEGF receptor activation may lead to Ras activation, and some involve RasGRP3, as follows. (i) Activation of PLC- γ leads to DAG production and RasGRP3 activation. (ii) DAG also recruits PKC which can phosphorylate RasGRP3. (iii) PKC may also activate Ras through SPK. (iv) VEGF signaling through SOS/Grb2 may lead to Ras activation. (v) DAG production can occur independently of VEGF signaling through aberrant glucose metabolism.

localized to the membrane, so developing ES cell-derived vessels were incubated with PMA, a phorbol ester that mimics DAG and is predicted to localize RasGRP3 to the membrane (47). RasGRP3-expressing wild-type vessels responded dramatically to PMA, exhibiting extensive morphogenetic changes that resulted in the formation of sheets rather than branched vessels. A preliminary analysis indicates that both increased endothelial proliferation and aberrant cell-cell interactions contribute to the phenotypic response to PMA (Roberts and Bautch, unpublished). PMA has complex effects on endothelial cells, since it also potently activates PKC and increases vascular permeability (39, 52, 75). However, despite the potential complexity of the endothelial response to PMA, it is dependent on RasGRP3, since *Rasgrp3^{gt/gt}* vessels were completely refractory to the PMA-induced morphogenetic changes. This result shows that RasGRP3 functions in endothelial cells and confirms that the *Rasgrp3^{gt}* allele is a loss-of-function mutation. Future work will delineate the components downstream of the response of newly formed vessels to PMA and how these components interact with RasGRP3.

How does RasGRP3 function in endothelial cells? One clue comes from the phenotype of wild-type vessels incubated with PMA, which in many aspects resembles the phenotype of vessels with up-regulated VEGF signaling. Although the VEGF receptor *flt-1* (VEGFR-1) can activate downstream signaling pathways in some contexts (82), *flt-1* is thought to act developmentally primarily as a decoy receptor to bind excess VEGF (42, 44, 59). Thus, deletion of *flt-1* in ES cell cultures leads to aberrant vascular morphogenesis and large endothelial sheets, and these perturbations are consistent with up-regulation of VEGF signaling (42). This phenotype results from both increased proliferation and compromised migration of affected endothelial cells (42, 44). RasGRP3 expression is up-regulated in the *flt-1* mutant ES cell cultures, consistent with the possibility that excess Ras activation through RasGRP3 contributes to the *flt-1* mutant phenotype. In addition, data suggests that RasGRP3 also activates other Ras-related GTPases such as Rap (55, 68, 78), so RasGRP3-mediated activation of Rap may also contribute to the vascular phenotype seen in the *flt-1* mutants and upon exposure to PMA. However, since PMA stimulation of primary endothelial cells results in robust Ras activation (75), Ras signaling is likely to contribute extensively to PMA-mediated vascular effects. Moreover, preliminary results indicate that the farnesyltransferase inhibitor FTI-2153 partially rescues the aberrant vascular phenotype induced by PMA (Roberts and Bautch, unpublished), a finding consistent with a model in which Ras is a downstream target of RasGRP3. Whether PMA stimulation also activates Rap in endothelial cells is yet to be determined. It will be interesting to determine which pathways are activated downstream of the *flt-1* mutation, and which components of VEGF signaling are mediated by Ras activation in vivo.

Our data demonstrate that endothelial cell expression of a GEF, RasGRP3, is downstream of VEGF signaling during blood vessel formation in vivo. RasGRP3 functions in endothelial cells, as it is required for PMA-induced vascular morphogenetic defects, but it does not appear to be essential for vascular development or normal angiogenesis. We suggest that this mode of Ras activation downstream of VEGF signaling is important in situations of pathway activation where redundant

pathways are not up-regulated. RasGRPs uniquely utilize DAG to localize to the membrane and activate Ras, and DAG is produced by several intracellular pathways. Thus, disease states that lead to excess DAG production may depend on RasGRP3-mediated Ras activation (Fig. 11). Several studies have correlated aberrant glucose metabolism and concomitant increases in DAG with diabetes (for reviews, see references 10 and 77). Thus, RasGRP3 may be involved in the vascular complications of diabetic patients, such as nephropathy and retinopathy, and it may provide a novel target for therapeutics aimed at ameliorating diabetic symptoms.

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