Vascular endothelial growth factor (VEGF) is an important mediator of ocular neovascularization by exerting its endothelial specific mitogenic effects through high affinity tyrosine kinase receptors. By screening a rat retina cDNA library, we have isolated a clone encoding the full-length prototypic form of the rat VEGF receptor-2/Flk-1, as well as a short form of the mRNA that encodes the complete seven N-terminal immunoglobulin-like extracellular ligand-binding domains, transmembrane region, NH₂-terminal half of the intracellular kinase domain, and kinase insert domain but does not encode the COOH-terminal half of the intracellular kinase domain and carboxyl-terminal region. Both forms of mRNA are detected in rat retina, although the short form is expressed at a lower level. VEGF induced a biphasic increase of cytoplasmic calcium with both forms in HK 293 transfected cells, indicating that both forms of the VEGF receptor-2/Flk-1 are functional and that the COOH-terminal half of the intracellular kinase domain and carboxyl region of VEGF receptor-2/Flk-1 are not strictly necessary for either ligand binding or this biological activity.

Vascular endothelial growth factor (VEGF) is a potent mitogen for vascular endothelial cells, both in vitro (1) and in vivo (2–4). VEGF exists as a homodimeric glycoprotein composed of one pair of four isoforms, generated by alternative splicing of the VEGF gene transcript (5, 6). There are two specific VEGF receptors, both of which are protein tyrosine kinases. VEGF receptor-1 (VEGFR-1), originally named Flt-1 (7), and VEGF receptor-2 (VEGFR-2), also known as Flk-1 and KDR (8, 9), are structurally related to each other, containing seven similar extracellular immunoglobulin domains and a conserved intracellular tyrosine kinase domain interrupted by a kinase insert domain (7, 10, 11). Through interactions with its receptors VEGFR-1/Flt-1 and VEGFR-2/Flk-1, VEGF appears to play a major role in angiogenesis during normal embryonic development (8, 12) and under pathological conditions such as diabetic retinopathy, rheumatoid arthritis, psoriasis, cardiovascular diseases, and tumor growth and metastasis (13).

Recent studies have provided evidence for a correlation of VEGF expression with retinal neovascularization in experimental models (14–17). Elevated levels of VEGF have been found in the vitreous of patients with proliferative diabetic retinopathy (18). It has also been demonstrated that several types of cultured retinal cells secrete VEGF and that hypoxia stimulates VEGF production (19–21). VEGF levels also increase in a primate model of iris neovascularization (22).

Although several studies have shown that VEGF is expressed and regulated in retinal cells, very little is known about the expression, regulation, and signal transduction mechanisms of the VEGF receptors in neural retina. The cDNAs for the VEGFR-2/Flk-1 have been cloned and characterized from humans (9, 11) and mice (8, 10, 23). However, the full-length cDNA sequence for the VEGFR-2/Flk-1 cloned from rat has not been reported.

In the current study, we have examined the expression pattern of VEGF receptor mRNA in different rat tissues. By isolating and characterizing the full-length cDNA sequences for VEGFR-2/Flk-1 from rat retina, we have found two forms of the receptor: a long form that is similar to the previously reported sequences for mouse and human, and a short form that has part of the intracellular tyrosine kinase domain deleted from the carboxyl terminus. Both forms of the receptor were expressed in mammalian cells and responded to VEGF with a rapid mobilization of calcium.

**Experimental Procedures**

**Probe Preparation by RT-PCR—**VEGFR-2/Flk-1 primers were designed from the conserved amino acid sequences ANEGELKT and DSITSSQ, located in the intracellular amino-terminal half of the tyrosine kinase domain and kinase insert region of mouse Flk-1 (10). The primer sequences used were MFlk2575S (5’-GCC AAT GAA GGA CTG AAG AC-3’) and MFlk3111AS (5’-CGT GCC CTT GGT GCT GTC C-3’). A ~500-bp RT-PCR product was amplified from 2 μg of rat retina total RNA and cloned into pCRII vector (Invitrogen, San Diego, CA). The cloned PCR product was sequenced on both strands by the chain termination method (24) using T7 and SP6 primers to confirm that the fragment was related to the VEGFR-2/Flk-1. This RT-PCR product was then used for cDNA library screening, Northern and Southern blot analysis, and in situ hybridization.

**Northern Blot Analysis—**To examine the expression of VEGFR-2 from various tissues, total RNA was isolated from adult rat brain, heart, kidney, liver, lung, skeletal muscle, retina, and spleen using the guanidinium thiocyanate-acid phenol method (25). Poly(A)+ RNA was prepared using Poly(A) Tract mRNA Isolation System (Promega, Madison, WI). Five μg of poly(A) RNA from each tissue was fractionated on 1% agarose-formaldehyde gels (26) and transferred to NYTRAN membranes (Schleicher & Schuell). The DNA probes were as follows: for rat VEGF-2/Flk-1, a 537-bp cDNA fragment generated from rat retina total RNA by RT-PCR with primers MFLK2575S and MFLK3111AS as described above; for rat β-actin, a ~700-bp fragment generated by RT-PCR with rat β-actin amplimer set (CLONTECH, Palo Alto, CA). Probes were radiolabeled with [α-32P]dCTP using a random primer.
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labeling kit (Stratagene, La Jolla, CA) to specific activities of 1–10 x 10⁶ cpm/µg. The blot was hybridized (27) with radiolabeled probe overnight at 65 °C in a buffer containing 0.45 M sodium phosphate (pH 7.2), 7% SDS, 1% bovine serum albumin, and 20 mM EDTA. The hybridized blot was washed finally with 0.1 x SSC, 0.1% SDS at 65 °C and autoradiographed for 4 h. The exposed films were scanned with a densitometer (Molecular Dynamics, Sunnyvale, CA). Hybridization with a rat β-actin cDNA was performed to check the quantity and quality of RNA present in each lane of the blots. Autoradiograms were analyzed by quantitative computing densitometer with ImageQuant (Molecular Dynamics, Sunnyvale, CA).

Screening of Rat Retina cDNA Library—The 537-bp rat VEGFR-2/Flk-1 cDNA fragment generated as described above was cloned into M13mp9 vector (Molecular Expressions, Sunnyvale, CA) and used as a probe to screen an oligo(dT)-primed rat retina cDNA library in the vector AZAP (Stratagene, La Jolla, CA) using standard procedures (26). Secondary and tertiary screening were performed until well isolated positive plaques were obtained. The pBlueScript phagemids with target insert were rescued from AZAP vector by in vitro excision using ExAssist/SOLR system (Stratagene, La Jolla, CA). The phagemid DNAs were isolated, and the size of the cloned inserts was determined by restriction digestion followed by agarose gel electrophoresis. DNA was sequenced from both directions with successive primers. Sequence analysis was performed with Hitachi MacDNAsis software (National Biosciences, Plymouth, MN).

Cloning of cDNA Ends by RACE—Since the VEGFR-2/Flk-1 cDNA inserts that were isolated from rat retina cDNA library lacked 5'-ends, we used the 5'-RACE (28) to determine the 5'-end sequence by using the Marathon cDNA amplification kit (CLONTECH). First-strand cDNA was synthesized from 1 µg of total poly(A)+ RNA with an oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase. Second strand cDNA synthesis was performed with a mixture of E. coli DNA polymerase I, RNase H, and E. coli DNA ligase. Following the creation of blunt ends with T4 DNA polymerase, the double-stranded cDNA was ligated to the Marathon cDNA adaptor by T4 DNA ligase. The 5'-RACE reaction was performed with the Marathon adaptor primer and gene-specific antisense primer RRFk1968AS, 5'-ATG TGG ACC GAT GTT GCC GTG GAT C-3', which was designed from the 5'-end sequence of the isolated cDNA insert. The PCR product was sequenced with (CLONTECH), which provides proof-reading activity, was used for the PCR reactions. The 5’-RACE PCR product was cloned into pCRII (Invitrogen) and characterized by DNA sequencing from both directions with successive primers.

Southern Blot Analysis—To confirm the existence of mRNA encoding the short form of VEGFR-2/Flk-1 in the retina, Marathon cDNA from the retina amplified with a pair of primers designated as RRFlk2610S (5'-GCC AAT GAA GGG GAA CTG AAG ACA-3') and RRFLk5155AS (5'-CCA GGG CAG ACA CAA GTT GGT AT-3'). The PCR product was electroeluted in an 12% agarose gel and transferred onto a NYTRAN membrane. The blot was hybridized with a 537-bp rat VEGFR-2/Flk-1 cDNA probe as described for the Northern blot analysis.

In Situ Hybridization—For in situ hybridization studies, eyes from normal adult rats were encased, fixed in 4% paraformaldehyde at 4 °C overnight, and embedded in paraffin. Serial 5-μm sections were cut and transferred to slides. Sense and antisense single-stranded RNA probes for the short form of VEGFR-2/Flk-1 in the rat retina, Marathon cDNA inserts that were isolated from rat retina cDNA library were radiolabeled with [α-35S]UTP in the presence of either T7 or SP6 RNA polymerase corresponding to the promoters flanking the insert sequences in pCRII (Promega). The blots were hybridized with a 537-bp rat VEGFR-2/Flk-1 cDNA probe as described for the Northern blot analysis. The blots were washed finally with 0.1× SSC, 0.1% SDS at 65 °C and autoradiographed for 15 days at −70 °C. The exposed films were scanned with a densitometer with ImageQuant (Molecular Dynamics). The hybridized blot was washed finally with 0.1× SSC, 0.1% SDS at 65 °C and autoradiographed for 4 h. The exposed films were scanned with a densitometer (Molecular Dynamics, Sunnyvale, CA). Hybridization with a rat β-actin cDNA was performed to check the quantity and quality of RNA present in each lane of the blots. Autoradiograms were analyzed by quantitative computing densitometer with ImageQuant (Molecular Dynamics, Sunnyvale, CA).

Expression of VEGFR-2/Flk-1 in Adult Rat Tissues—RT-PCR was used to amplify a DNA fragment corresponding to the kinase insert domain of VEGFR-2/Flk-1 from rat retina total RNA with a pair of primers designed from the mouse Flk-1 sequence (10). The 537-bp PCR product was subcloned into a plasmid vector pCRII and sequenced. Computer-assisted comparison with mouse Flk-1 DNA sequence (10) revealed a 93% identity, which suggests that this DNA fragment represents the rat version of the VEGFR-2/Flk-1 gene. This DNA fragment was used to examine the expression pattern of VEGFR-2/Flk-1 in normal adult rat tissues by Northern blot analysis (Fig. 1). The VEGFR-2/Flk-1 mRNA was detected in several tissues including brain, heart, kidney, liver, lung, skeletal muscle, retina, and spleen with a major band at about 6 kb (Fig. 1A). The expression level for most tissues was low. However, the mRNA expression of VEGFR-2/Flk-1 in retina was relatively abundant, at a level as high as that in lung, which has been reported as a tissue with high expression of this gene (10, 23). A smaller band was also detected in retina and lung at about 4 kb (Fig. 1A).

Cloning and Characterization of Rat VEGFR-2/Flk-1 cDNA—Three independent positive clones were found using a 537-bp rat VEGFR-2 cDNA fragment as probe to screen 5.4 x 10⁶ recombinants from a rat retina cDNA library. They are designated as RRFLk1429, RRFLk3600, and RRFLk2053, respectively, based on their size (Fig. 2). Partial DNA sequencing of both ends revealed the presence of poly(A)+ tails. Sequence analysis was performed with Hitachi MacDNAsis software (National Biosciences, Plymouth, MN).

Expression of VEGFR-2/Flk-1 mRNA with a pair of primers designed from the mouse Flk-1 sequence (10). The 537-bp PCR product was subcloned into a plasmid vector pCRII and sequenced. Computer-assisted comparison with mouse Flk-1 DNA sequence (10) revealed a 93% identity, which suggests that this DNA fragment represents the rat version of the VEGFR-2/Flk-1 gene. This DNA fragment was used to examine the expression pattern of VEGFR-2/Flk-1 in normal adult rat tissues by Northern blot analysis (Fig. 1). The VEGFR-2/Flk-1 mRNA was detected in several tissues including brain, heart, kidney, liver, lung, skeletal muscle, retina, and spleen with a major band at about 6 kb (Fig. 1A). The expression level for most tissues was low. However, the mRNA expression of VEGFR-2/Flk-1 in retina was relatively abundant, at a level as high as that in lung, which has been reported as a tissue with high expression of this gene (10, 23).

A smaller band was also detected in retina and lung at about 4 kb (Fig. 1A).
indicated that the three clones shared the same 3' -end of about 800 bp containing poly(A'), but none of the clones reached the complete 5' -end of the Flk-1 cDNA sequence when compared with the cDNA sequence data from other species (8–11). To determine the 5' -end sequence of rat retina VEGFR-2/Flk-1 cDNA, 5' -RACE was performed with the antisense primer RRFlk1996AS. From this, a 2020-bp cDNA fragment was generated and cloned. As a result of sequencing and comparing the overlapping clones, the complete cDNA sequence for rat retina VEGFR-2/Flk-1 was found to contain 5892 nucleotides (GenBank™ accession number U93306). An open reading frame of 4029 nucleotides is flanked by 248 nucleotides of 5' -untranslated sequence and 1615 nucleotides of 3' -untranslated sequence. The presence of a consensus polyadenylation signal at nucleotide 5858 suggests that the 3' -noncoding region is complete. The first ATG codon found in this open reading frame is in good agreement with the consensus sequence for translation initiation (33). The deduced 1343-amino acid polypeptide is 24 and 11 residues shorter than mouse and human VEGFR-2/Flk-1 (9–11) (Fig. 3A). Overall, it shows 69.2 and 51.2% identity in nucleotide sequence and 95 and 87% identity in deduced amino acid sequence to its mouse and human homologues. Fig. 3B shows that there is a high degree of sequence conservation in the structure of VEGFR-2/Flk-1 at the amino acid level among rats, humans, and mice.

Cloning of Short Form of the VEGF Receptor-2/Flk-1—On the basis of sequence analysis, one of the three clones isolated from a retina cDNA library, RRFlk3600, appears to be derived from the prototypic form of VEGFR-2/Flk-1, whereas the other two clones encode a short form of the VEGFR-2/Flk-1 with a truncated COOH terminus. The two truncated VEGFR-2/Flk-1 clones are also independent of each other because they terminate at different 5' -sites. The shorter 1.4-kb clone (RRFlk1429) begins at nucleotide 2588, whereas the longer 2.0-kb clone (RRFlk2053) begins at nucleotide 1964 of the full-length prototypic form of the rat VEGFR-2/Flk-1 sequence (GenBank™ accession number U93306). The clone RRFlk2053 spans the coding sequence from the sixth immunoglobulin-like domain to the 3' -poly(A') end. The cDNA coding sequence of both of the truncated VEGFR-2/Flk-1 clones are identical to the full-length prototypic form up to nucleotide 3222, at which the open reading frame is terminated by TGA (Fig. 4A). The complete cDNA sequence for the short form of rat VEGFR-2/Flk-1 was obtained by lining up the overlapping 2020-bp 5' -RACE product (described above) and clone RRFlk2053, which contains 4016 nucleotides (GenBank™ accession number U93307). An open reading frame of 2973 nucleotides encoding 991 amino acids is initiated by an ATG codon at nucleotide 249. A deletion of 1876 nucleotides (1082 nucleotides for the coding sequence and 794 nucleotides for the noncoding sequence) from the rat prototypic form of VEGFR-2/Flk-1 mRNA results in the deletion of 352 amino acids from the COOH-terminal half of the intracellular kinase domain and carboxyl-terminal region in the short form of rat VEGFR-2/Flk-1 (Fig. 4B).

The expression of both long and short forms of VEGFR-2/Flk-1 in rat retina was confirmed by RT-PCR with primers (RRFlk2610S and RRFlk5155AS) spanning the deletion site and common to both forms of the receptor, followed by Southern blot analysis with a 537-kb DNA probe corresponding to the kinase insert domain, which is present in both forms of the receptor. Two bands (2545 bp for the long form and 669 bp for the short form of VEGFR-2/Flk-1) were produced by RT-PCR using rat retina poly(A') RNA (Fig. 4C). The Southern blot in Fig. 4D shows that both bands were specific to VEGFR-2/Flk-1. The 669-bp band was very weak in the ethidium bromide-stained gel due to the low level of expression of the short form VEGFR-2/Flk-1 in rat retina, but it was easily visualized after Southern hybridization. No PCR product was detected in control reactions without RT (data not shown), eliminating the possibility of contamination by genomic DNA.

Location of VEGFR-2/Flk-1 mRNA in Retina—To determine
the location of VEGFR-2/Flk-1 mRNA expression in rat retina, in situ hybridization was performed. Fig. 5 shows the results of a representative in situ hybridization performed in normal adult rat retina. Hybridization with an antisense probe specific to both the long and short forms of VEGFR-2/Flk-1 shows an intense signal for VEGFR-2/Flk-1 mRNA in the inner nuclear layer of retina and also some labeling over choroid (Fig. 5, A and B). A similar distribution pattern was obtained with the antisense probe specific to only long form of VEGFR-2/Flk-1 except that less labeling was found in the boundary of inner nuclear layer and inner plexiform layer (Fig. 5, E and F), compared with the same area in Fig. 5, A and B. Hybridization with sense probes shows low background in most areas of retina (Fig. 5, C, D, G, and H), with the exception of the photoreceptor outer segments, which showed homogenous non-specific labeling with both antisense and sense probes (Fig. 5, B, D, F, and H), and thus the signal over this area does not imply the presence of VEGFR-2/Flk-1 transcripts. Closer examination with high power magnification in light field microscopy (Fig. 6) clearly shows that the dark grains denoting VEGFR-2/Flk-1 mRNA/probe hybrids are located in the inner nuclear layer of the retina with high density for both antisense probes (Fig. 6, A and C, down arrows). Significant labeling can be found in the choroid for both antisense probes (Fig. 6, A and B, and C, down arrows).
Expression of Both Forms of VEGFR-2—The full-length coding sequences for both long and short forms of VEGFR-2/Flk-1 generated from RT-PCR were cloned into the pcDNA3 vector, which contains a CMV early promoter region upstream of the multiple cloning site. To confirm that the open reading frame sequences present in the RRFlk-L/pcDNA3 and RRFlk-S/pcDNA3 clones code for translatable proteins, the cDNAs from each construct were transcribed using T7 polymerase and translated in rabbit reticulocyte lysate. The translated product from the RRFlk-L/pcDNA3 clone was a protein of ~110 kDa. In the presence of microsome membrane to allow glycosylation, the molecular mass shifted for both constructs to ~190 and ~145 kDa, respectively. Multiple protein translation products with smaller molecular mass were also found for both constructs, most probably because of the in-frame ATGs in the nucleotide sequences.

The RRFlk-L/pcDNA3 and RRFlk-S/pcDNA3 were transfected transiently into HK 293 cells using a calcium precipitation method. VEGFR-2/Flk-1 mRNA production was assessed by Northern blot analysis using the 537-bp VEGFR-2/Flk-1 (E and F; down arrows indicate the expression in the inner nuclear layer, and up arrows indicate the expression in the border of the inner nuclear layer/inner plexiform layer), antisense RNA probe specific to only the long form of VEGFR-2/Flk-1 (G and H) and photographed with light (A, C, E, and G) and dark field (B, D, F, and H). Note the background bright signal in the photoreceptor outer segments for both antisense and sense probes in dark field (B, D, C, and D). The choroid (CH), photoreceptor outer segments (POS), outer nuclear layer (ONL), inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GCL) are indicated (magnification × 200.)
were found with sizes of rat retina was loaded on the gel, and two hybridized bands A (Fig. 9 pcDNA3 alone. As a positive control, 2 signal was found from control HK 293 cells transfected with frames for the long and short forms of VEGFR-2/Flk-1. No band was found after transfection with RRFlk-S/pcDNA3 (Fig. 5). These bands correspond to the expected size of open reading segment, outer nuclear layer (ONL), inner nuclear layer (INL), and inner plexiform layer (IPL) are indicated.

**Functional Response of the Expressed Receptor**—The functional expression of the receptors was examined by measurement of a calcium signal in transfected HK 293 cells after the addition of recombinant human VEGF165. The imaging system allowed calcium responses to be measured from several cells simultaneously. Approximately 20% of the HK 293 cells transfected with RRFlk-L/pcDNA3 responded to 1 nM VEGF165 (Fig. 9A), whereas those HK 293 cells transfected with RRFlk-S/pcDNA3 showed slightly more cells (25%) responding (Fig. 9B). The response consisted of a rapid increase in intracellular calcium (~500 nM) with a return to near base line in ~1 min. No response of intracellular calcium was observed in HK 293 cells transfected with pcDNA3 alone (Fig. 9C). Similar calcium responses were obtained when 1 nM VEGF121 was used to stimulate HK 293 cells transfected with either RRFlk-1/pcDNA3 or RRFlk-S/pcDNA3 (data not shown). To further demonstrate unequivocally that the VEGF-induced responses of intracellular calcium in the transfected HK 293 cells were specifically due to VEGF binding, an anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used to neutralize VEGF activity. Incubation of VEGF with excess soluble anti-VEGF antibody (10 μg/ml) completely inhibited the VEGF activity upon inducing response of intracellular calcium in HK 293 cells transfected with either the long or short form of VEGFR-2/Flk-1 cDNAs (Fig. 9, A and B).

**DISCUSSION**

In the present study, we have cloned, sequenced, and expressed two variant cDNA clones for VEGFR-2/Flk-1 from a rat retina cDNA library. They code for two distinct forms of VEGFR-2/Flk-1, which represents the first report of the variant for the VEGFR-2/Flk-1. The long form, RRFlk-L, encodes...
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the prototypic form of VEGFR-2/Flk-1 that includes a hydrophobic leader sequence following the initiator methionine; the extracellular domain containing seven Ig-like domains (the fourth of which does not contain cysteine residues); a transmembrane region; an intracellular kinase domain that is interrupted into an NH₂-terminal half and a COOH-terminal half by a kinase insert domain; and a carboxyl-terminal region (Fig. 3A). The short form, RRFlk-S, encodes a truncated form of VEGFR-2/Flk-1, which is identical to the long form, except for a deletion of the COOH-terminal half of the kinase domain and carboxyl region in the intracellular portion.

The cDNA encoding RRFlk-S was found in two of three independent RRFlk clones, so the corresponding mRNA is neither an artifact of cloning nor likely to be rare. The expression of the short form of VEGFR-2/Flk-1 (RRFlk-S) in rat retina was confirmed by RT-PCR with a pair of primers spanning the deletion site, followed by Southern blot with the probe corresponding to the kinase insert domain (Fig. 4). Additional evidence also came from Northern blot analysis when we increased the amount of poly(A⁺) RNA up to 5 µg loaded on the gel, which shows two bands at ~6 and ~4 kb corresponding to mRNAs encoding the long and short forms of VEGFR-2/Flk-1, respectively, in rat lung and retina (Fig. 1A). The short form of VEGFR-2/Flk-1 is expressed at a very low level, because it was difficult to detect the ~4-kb band from any tissue examined in the same condition when 1 µg of poly(A⁺) RNA was loaded on the gel (data not shown). The RRFlk-L and RRFlk-S are probably alternatively spliced variants of a single gene, because the 3’-terminal tail of RRFlk-S (nucleotides 3223–4016) is found to be exactly identical to the extreme 3’-untranslated region of RRFlk-L (nucleotides 5099–5898). However, the splice site cannot be determined without the genomic structure data of VEGFR-2/Flk-1.

The identification of splice variants among the tyrosine kinase receptors has been increasing at a phenomenal rate. Splice variants have been identified for the Eph-related receptor tyrosine kinase Cek9 (34); fibroblast growth factor receptor, SpFGFR1 (35); neurotrophin receptor TrkB (36); the receptor for colony-stimulating factor 1, FLT3 (37); hepatocyte growth factor receptor, c-met (38); and nerve growth factor receptor, TrkA (39, 40). Furthermore, Kendall and Thomas (41) reported the existence of a cDNA encoding a truncated form of VEGF receptor-1/Flt-1 lacking the seventh immunoglobulin-like domain, the transmembrane sequences, and the entire cytoplasmic domain in human umbilical vein endothelial cells. It was hypothesized that this variant might encode a soluble form of VEGF receptor that acts as a specific antagonist of VEGF. However, it is interesting to note that in the short form of VEGFR-2/Flk-1 the cytoplasmic domain of the receptor is truncated, indicating that alternative RNA splicing may play an important role in the generation of functionally divergent receptor activities.

In situ hybridization studies show that VEGF-2/Flk-1 mRNA is highly expressed by cells in the inner nuclear layer of the retina (Figs. 5 and 6). This is consistent with a prior report that Flk-1 transcripts were expressed in a row of cells within the inner nuclear layer of retina in adult mice (42). Our in situ hybridization studies also show that VEGF-2/Flk-1 transcripts are expressed in choroid of the retina (Figs. 5 and 6). Since the RRFlk-L shares all of the sequence information that RRFlk-S has, it is difficult to precisely distinguish the difference in location between these two forms of the VEGF-2/Flk-1 transcript in the retina. To localize mRNA expression of the two forms of VEGF-2/Flk-1 in the retina, we used two different antisense RNA probes for in situ hybridization; one is specific to both RRFlk-L and RRFlk-S, and the other is specific only to RRFlk-L. Comparison of the in situ hybridization results (Figs. 5 and 6) with two different probes suggests that RRFlk-S appears to be expressed by the cells in the border between the inner nuclear layer and inner plexiform layer of retina, where RRFlk-L is less expressed. However, this does not exclude the possibility that RRFlk-S is also expressed in the inner nuclear layer and choroid where RRFlk-L is expressed.

To functionally characterize the cDNA encoding for VEGFR-2/Flk-1 isolated from rat retina, we subcloned the full-length coding sequences for both RRFlk-L and RRFlk-S into the expression vector pcDNA3 and transiently expressed them in HK 293 cells. The in vitro transcription and translation (Fig. 7) clearly indicated the presence of translatable, functional coding sequences. The Northern blot analysis after 48 h of transfection (Fig. 8) indicated that both of RRFlk-L and RRFlk-S mRNA were well expressed. It has been reported that VEGF induces a rapid Ca²⁺ entry into cultured endothelial cells from bovine aorta, human umbilical vein, and bovine adrenal cortex (43). The measurement of cytoplasmic calcium has been considered the most sensitive assay currently available for detecting VEGF activity (44), and it has been used in several studies including the biological activation of VEGF receptor-1/Flt-1 (7) and hypoxia-induced VEGF gene expression (45). We used a similar technique to examine the functional response of the expressed VEGF receptors. The addition of VEGF to the HK

**Fig. 9. Functional response of the expressed VEGF-2/Flk-1 by image analysis of intracellular calcium.** The change of intracellular calcium [Ca²⁺], in single HK 293 cells transfected with RRFlk-L/pCNA3 (A), RRFlk-S/pCNA3 (B), and pCNA3 (C) after 48 h of transfection was observed using a video imaging system. HK 293 cells were loaded with the fluorescent Ca²⁺ indicator Fura-2/AM (3 µM), and the fluorescent signal measured with 340- and 380-nm excitation was used to calculate [Ca²⁺], for each cell. The addition of VEGF (1 nM) is indicated by the **arrow**.
293 cells induced a 6–7-fold increase in intracellular calcium in HK 293 cells transfected with either RRFK1-pcDNA3 or RRFK1-S-pcDNA3 (Fig. 9, A and B). Mock transfection of HK 293 cells with pcDNA3 did not respond to VEGF (Fig. 9C). These findings show that VEGF can induce a specific functional response of both long and short forms of VEGFR-2/Fk-1 encoded by cDNAs isolated from rat retina. They also indicate that VEGF is a ligand for both receptors and that the COOH-terminal half of the kinase domain and carboxyl region of the VEGF receptor-2/Fk-1 (amino acids 992–1343) are not required for this biological response. Further studies will therefore be required to determine if there is a functional difference between the two receptor variants.

The Northern blot data indicates that the VEGF-2/Fk-1 is expressed in the retina of normal adult rat at a relatively high level compared with other tissues examined (Fig. 1). In situ hybridization in adult rat retina shows that VEGF-2/Fk-1 is expressed in the cells of inner nuclear layer and choroid (Figs. 5 and 6). In situ hybridization in mouse retina (42) has detected the VEGF-2/Fk-1 expression in the neural retina during mouse embryogenesis and in the retinal Muller glial cell adult of mouse. The Muller cells are radially aligned and span most of the thickness of the retina and play an important role in maintaining the extracellular homeostasis of ions, metabolites, and signaling molecules. The ligand for VEGF-2/Fk-1, VEGF, is likewise expressed in Muller glial cells (17) at a relatively high level in the retina and has been proposed to play a role in vascular permeability and angiogenesis (44). Since VEGF-2/Fk-1 expression appears not to be restricted to retinal endothelial cells, it is tempting to speculate that the high level of expression for VEGF and VEGF-2/Fk-1 in the retina might reflect an alternative function for this ligand and receptor. Since VEGF is secreted by the retina under normal conditions, VEGF-2/Fk-1 on Muller cells may function as a specific uptake mechanism and regulator of the extracellular concentration of this potent signaling protein.

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