Genomic Organization of the Mouse and Human Genes for Vascular Endothelial Growth Factor B (VEGF-B) and Characterization of a Second Splice Isoform*

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A second isoform and the genomic structures of mouse and human vascular endothelial growth factor B are described. Both genes consist of seven coding exons and span about 4 kilobases of DNA. The two identified isoforms of vascular endothelial growth factor B are generated by alternative splicing where different splice acceptor sites in exon 6 introduce a frameshift and a partial use of different but overlapping reading frames. Consequently, the COOH-terminal domains in the two isoforms show no resemblance. Mouse and human cDNA clones for the novel isoform of vascular endothelial growth factor B encoded a secreted protein of 186 amino acid residues. Expression in transfected cells generated a protein of 25 kDa which upon secretion was modified by O-linked glycosylation and displayed a molecular mass of 32 kDa under reducing conditions. The protein was expressed as a disulfide-linked homodimer, and heterodimers were generated when coexpressed with vascular endothelial growth factor. The entirely different COOH-terminal domains in the two isoforms of vascular endothelial growth factor B imply that some functional properties of the two proteins are distinct.

The platelet-derived growth factor (PDGF)1/vascular endothelial growth factor (VEGF) family comprises at present six different members, i.e. PDGF-A and -B (1), VEGF (2), VEGF-B (3), VEGF-C (4, 5) and placenta growth factor (PlGF) (6). These growth factors are secreted as dimers and share a number of structural and functional properties. They display 20–45% amino acid sequence identity including eight invariant cysteine residues involved in intra- and intermolecular disulfide bonding of the two subunits. The COOH-terminal coding regions in several of these growth factors vary by alternative splicing, giving rise to distinct isoforms. It is conceivable that the different biochemical properties of the isoforms may regulate their cellular release, compartmentalization, and bioavailability, and possibly also modulate the signaling properties of these growth factors.

Specific receptor tyrosine kinases for this class of growth factors are involved in the regulation of a number of processes in the target cells. The dimeric growth factors induce receptor dimerization that initiates cellular signaling via the intracellular kinase domains of the receptors (7). PDGF-A and -B are recognized by PDGF-a and -b receptors (8–11). VEGF is recognized by the Flk-1/KDR and Flt-1 receptors (12–14), PlGF by the Flt-1 receptor (15), and VEGF-C by the Flt-4 and Flk-1/KDR (4, 5). The receptor(s) for VEGF-B remains to be identified.

VEGF is an endothelial cell-specific mitogen that acts as a potent angiogenic factor. In addition, it induces blood vessel permeability (16). Four splice isoforms of human VEGF have been described (17–19), whereas the mouse apparently expresses only three isoforms (20). The longer isoforms of VEGF are highly basic and bind to cellular or pericellular heparan sulfate proteoglycans, whereas the shorter isoforms are soluble in the extracellular fluid (17–19). PlGF is closely related to VEGF, but its functional role is obscure at present. However, similarly to VEGF, different splice isoforms of PlGF have been identified (6, 21, 22).

The specific mechanisms regulating angiogenesis are poorly understood, but it is conceivable that receptor tyrosine kinases and their ligands have important functional roles in the regulation of growth and differentiation of endothelial cells (for review, see Ref. 23). VEGF has been considered to be the major factor involved in normal and pathological angiogenesis (for review, see Ref. 24). These processes include embryonic development, wound healing, tissue regeneration and reorganization, growth and metastasis formation of solid tumors, and ischemia-induced retinal disorders. The importance of VEGF in the development and maintenance of the vascular tree was implicated further by the analysis of mice carrying targeted mutations in the two VEGF receptors, Flt-1 and Flk-1. Both mutations were found to be lethal. Embryos lacking functional Flk-1 lacked blood islands and hematopoietic precursor cells in the yolk sac and failed to develop a proper vasculature during early development (25), whereas embryos lacking functional Flt-1 formed endothelial cells but developed an abnormal organization of the vascular endothelium (26).

Recently, we identified a novel growth factor for endothelial cells, VEGF-B, which is expressed abundantly in cardiac and skeletal muscle (3). The isolated mouse and human cDNA clones for VEGF-B were found to encode secreted proteins of 167 amino acids (VEGF-B167) with strong affinity for heparin sulfate. Amino acid sequence comparisons showed that
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VEGF-B shared ~45% identity with VEGF and a lower degree of identity with PIGF and the two PDGF polypeptides.

In this work we have identified mouse and human cDNA clones encoding a novel isoform of VEGF-B consisting of 186 amino acids. The NH2-terminal domains of the two isoforms were identical, whereas the COOH-terminal portions showed no obvious similarities. Analysis of mouse and human genes for VEGF-B showed that the two isoforms were generated by alternative splicing of mRNA from a single gene. Thus VEGF-B, like most other identified growth factors in the PDGF/VEGF family, is expressed as distinct isoforms generated by alternative splicing.

EXPERIMENTAL PROCEDURES

Isolation of Human and Mouse cDNA Clones Encoding VEGF-B186—Several cDNA clones encoding mouse VEGF-B186 were isolated from an adult mouse heart ZAP II cDNA library as described previously for the isolation of mouse VEGF-B167 cDNA clones (3). The inserts of the cDNA clones were subcloned by in vivo excision, and both strands of the clones were fully sequenced.

Approximately 1 × 10⁶ clones of a human HT1080 fibrosarcoma cell cDNA library in Agt11 were screened using the same the probe (3). A positive clone, termed H.1, was analyzed and found to represent cDNA encoding VEGF-B186. Throughout this work standard molecular biology techniques were used (27).

Characterization of Mouse and Human Genes Encoding VEGF-B—Several DNA clones for the mouse VEGF-B gene were isolated from a 1295W F1X genomic library as recommended by the supplier (Stratagene). The same probe as above was used. The positive α clone 10 was subcloned as BamHI fragments into pBluescript SK (Stratagene). Isolated DNA from clone 10 was also used as the template in PCRs (100 ng of α DNA/reaction), and the coding parts of the mouse VEGF-B gene were amplified using different combinations of primers derived from the cDNA clones encoding VEGF-B186 and VEGF-B167. Taq DNA polymerase (2.5 units/reaction) was used. The generated PCR fragments were cloned into the TA-cloning vector pCR II (Invitrogen). The exon-intron structure of the mouse VEGF-B gene was established by nucleotide sequence analysis of the subcloned BamHI genomic fragments and of the cloned PCR products.

A human genomic DNA clone was isolated by screening 1 × 10⁶ clones of a human genomic library in EMBL-3 SP6/T7 (Clontech) using high stringency conditions with a 32P-labeled 90-bp PCR fragment spanning 5′-sequences of human VEGF-B cDNA as the probe. Primers used for the PCR were: 5′-CACATGAGCCCTGTGCTCC (forward) and 5′-GGGCATGCTTGAGGACAGCAG (reverse). The positive α clone 10 was subcloned as SacI fragments into pGem 3Z vector (Promega) and was found to carry the 5′-region of the gene. The remaining parts of the human VEGF-B gene were amplified by PCR using genomic DNA as the template. Different combinations of primers derived from the human cDNA clones of the two VEGF-B isoforms were used. Dynazyme DNA polymerase (2.5 units/reaction, Finnzymes) was used. The amplified PCR fragments were cloned into the TA-cloning vector pCR II. The exon-intron boundaries and the length of the short introns of the mouse and human VEGF-B genes were determined by nucleotide sequence analysis. The length of the larger introns was calculated based upon the length of the amplified PCR fragments when analyzed by agarose gel electrophoresis.

RT-PCR Analysis of VEGF-B Transcripts—Total RNA from mouse and human tissues was isolated using standard procedures (28). Two or 5 μg of total RNA/reaction was used for first strand cDNA synthesis using avian myeloblastosis virus reverse transcriptase (20 units/reaction). The reactions were primed with oligo(dT)18. Aliquots of these reactions were used as templates in PCRs using Taq DNA polymerase (2.5 units/reaction) was used. The generated PCR fragments were cloned into the TA-cloning vector pCR II and analyzed by nucleotide sequence analysis.

To amplify mouse cDNA two pairs of primers were used. These pairs were obtained by combining a common forward primer, 5′-CACAGCGCAATGTGAATGCA (mp1), located in exon 4, with two different reverse primers, 5′-GCTTCAGGCCCGCCCAGTAGGAGA (mp2) and 5′-ACGTAGATCTCCTTTGCCCCTGGCCCGCATCCTTCC (mp3) this primer is a BglII site and 4 extra bases in the 5′-end, located in exons 6B and 7, respectively. Following the analysis by agarose gel electrophoresis, the amplified bands were transferred onto a nylon filter (GeneScreen Plus) and sequentially hybridized with oligonucleotide probes specific for exon 6A and 6B. These [32P]dCTP end-labeled oligonucleotide probes were 5′-CTCTGTCCGGGCTGGGACTCTA (exon 6A) and 5′-TCAAGGGCGTTCAGGGCGCTGGTGC (exon 6B).

FIG. 1. Deduced amino acid sequences of mouse and human VEGF-B186 and a comparison with mouse and human VEGF-B167. Panel A, alignment of the amino acid sequences of mouse and human VEGF-B186 and VEGF-B167 isoforms. Residues identical to mouse VEGF-B186 are boxed. The arrow indicates the putative cleavage site for the signal peptidase. Panel B, hydrophilicity profiles of mouse VEGF-B186 and VEGF-B167 isoforms. The profiles were generated according to Kyte and Doolittle (35) using a window of nine residues.

were carried out at 37°C in 6 × SSC containing 5 × Denhardt's solution, 0.5% SDS, and 100 μg/ml salmon sperm DNA. The filters were washed at the same temperature in 6 × SSC containing 0.5% SDS twice for 15 min each and subjected to autoradiography.

The two pairs of primers used for amplification of human cDNA were combined using two different 5′-primers, 5′-CCTGACAGTGAGGAGTCTGTG (hp1, forward), located in exon 3, and 5′-TGTCCTGAGGAA-GAACCAGCCC (hp2, forward), located in exon 4, with a common 3′-primer, 3′-GCCATGTCCTGTCCTGCAG (hp3, reverse), located in exon 7. Alliquots of the amplified products were analyzed by agarose gel electrophoresis, and the amplified bands were cloned in the TA-cloning vector pCR II and analyzed by nucleotide sequencing.

Northern Blot Analysis of VEGF-B186 Transcripts—Adult mouse and human multiple tissue Northern blots (MTN, Clontech) were hybridized with a single-stranded DNA probe specific for exon 6A. The probe, labeled using a PCR-based strategy mainly as described (29). The primers 5′-GGTGTCAGCTTCCCAACCC (forward) and 5′-AGTGATGTCCTG (reverse) were used to amplify a 91-nucleotide fragment of murine VEGF-B186 exon 6A. The overnight hybridizations were carried out in 37% formamide, 5 × SSPE, 10 × Denhardt's solution, 2% SDS, and 100 μg/ml single-stranded salmon sperm DNA at 42°C. The filters were washed briefly in 2 × SSC containing 0.05% SDS at room temperature and then washed at 45°C in 0.1 × SSC containing 0.1% SDS. As a specificity control, EcoRI-out plasmids (1 μg of DNA) encoding mouse VEGF-B167 and VEGF-B186 were fractionated by agarose gel electrophoresis, blotted onto a filter, and hybridized and washed together with the multiple tissue Northern blot filters. The
filters were subjected to autoradiography.

Transfection and Analysis of COS-1 Cells Expressing VEGF-B186—
cDNA inserts encoding mouse VEGF-B186 and human VEGF165 were
cloned into the pSG5 expression vector (30). COS-1 cells were main-
tained in Dulbecco's modified Eagle's medium containing 10% fetal calf
serum, 2 mM glutamine, and appropriate antibiotics. The cells were
transfected with the expression vectors, separately or in combination,
using calcium phosphate precipitation. Following 36–48 h of incuba-
tion, the cells were incubated in methionine- and cysteine-free medium
for 30 min and then incubated in the same medium containing 100
mCi/ml [35S]methionine and [35S]cysteine for 2 h (Promix Amersham
Inc.) and then chased for an additional 2 h in minimal essential me-
dium. In some experiments 100 μg/ml heparin was added during the 2-h
chase period. Media were collected after the chase period, and the cells
were solubilized in 10 mM Tris buffer, pH 7.5, containing 50 mM NaCl,
0.5% deoxycholate, 0.5% Nonidet P-40, 0.1% SDS, and 1 mM phenyl-
methylsulfonyl fluoride. Aliquots of the media and of the cell lysates
were subjected to immunoprecipitation using specific antisera to mouse
VEGF-B and to human VEGF. The precipitates were analyzed by 10 or
15% SDS-PAGE. The dried gels were subjected to autoradiography. The
antisera to mouse VEGF-B were raised by immunizing rabbits with a
18-mer oligopeptide, comprising the NH2-terminal region of processed

![Exon-intron structures of the mouse and human VEGF-B genes](image)

**Fig. 2.** Exon-intron structures of the mouse and human VEGF-B genes. The exon sizes in base pairs are marked inside the boxes, and the
sizes of the introns are marked in between the boxes. The introns are not shown in scale. The alternative usage of acceptor sites in E6 generates
the two VEGF-B isoforms. The position of the stop codon (TAG) for VEGF-B186 is marked in E6B, and the stop codon (TGA) for VEGF-B167 is
marked in E7. The structures of the untranslated flanking regions of mouse and human VEGF-B genes were not established (gray boxes).

| Exon (bp) | Donor site | Intron length (bp) | Acceptor site |
|-----------|------------|--------------------|---------------|
| Mouse     |            |                    |               |
| E1 60     | T GCC ACC CAG/gtacggtcgtg | =590              | ttcgccacgg/GCC CCT GTC T |
| E2 43     | CAG AAG AAA /gtaaatag      | 287               | ctgccacgg/TG GTG CCA TG |
| E3 197    | C GGA ATG CAG/gtacgaggcc  | 161               | ctggacagcg/ATC CTC ATG A |
| E4 74     | GT GAA TGC AG/gtacgagc    | 178               | cttttttcg/A CCA AAA AAA |
| mVEGF-B186|            |                    |               |
| E5 36     | AG CCA GAC AG/gtggagttttt | =200              | cttcctctcg/G GTG CCC ATA |
| E6A 211   | stop codon in exon 6 (TAG) |                   | g Val Ala Ile |
| mVEGF-B167|            |                    |               |
| E5 36     | AG CCA GAC AG/gtggagttttt | =200              | cccactccag/C CCC AGG ATA |
| E6B 135   | AC ACC TGF AG/gtggagtttc | 2.6 kb            | ccctccccag/G TGC CGG AAC |
| E7 19     | Stop codon in exon 7 (TAG) |                   | g Cys Arg Lys |
| Human     |            |                    |               |
| E1 60     | C CCC CCC CAG/gtacgctcgg  | =760              | ttcgccacgg/GCC CCT GTC T |
| E2 43     | CAG AAG AAA /gtaaatctca   | 275               | ctggacagcg/TG GTG CCA TG |
| E3 197    | C GGA ATG CAG/gtacgaggcc  | 244               | ctggacagcg/ATC CTC ATG A |
| E4 74     | GT GAA TGC AG/gtacgagc    | 170               | cttttttcg/A CCT AAA AAA |
| hVEGF-B186|            |                    |               |
| E5 36     | AG CCA GAC AG/gtggagttttt | =200              | cttcctctcg/G GCT CCC ACT |
| E6A 211   | Stop codon in exon 6 (TAG) |                   | g Ala Ala Thr |
| hVEGF-B167|            |                    |               |
| E5 36     | AG CCA GAC AG/gtggagttttt | =200              | cccactccag/C CCC AGG CCC |
| E6B 135   | AC ACC TGF AG/gtggagttgg | 736               | ccctccccag/G TGC CGG AAC |
| E7 19     | Stop codon in exon 7 (TAG) |                   | g Cys Arg Lys |

**Fig. 3.** Nucleotide sequences of the exon-intron junctions in the mouse and human VEGF-B genes. Coding sequences are in upper case
letters; intron sequences are in lower case.

![Nucleotide sequences of the exon-intron junctions in the mouse and human VEGF-B genes](image)
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**RESULTS**

Primary Structure of a Second VEGF-B Isoform, VEGF-B<sub>186</sub>—Several cDNA clones isolated from an adult mouse heart cDNA library and the clone H.1 isolated from a human fibrosarcoma cDNA library differed in their nucleotide sequences from those cDNA clones previously found to encode VEGF-B<sub>167</sub> (3). Common to the mouse and human cDNA clones was an insertion of 101 bp found between nucleotides 410 and 411 in the coding sequence of VEGF-B<sub>167</sub>. Downstream of the insertion, the nucleotide sequences of these cDNA clones were identical to the VEGF-B<sub>167</sub> cDNA sequence. The position of the 101-bp insertion corresponded to the exon 5-exon 6 junction in VEGF (18–20), and it resulted in a shift of the reading frame of the translated nucleotide sequence downstream of the insertion and a stop codon (TAG) at a position corresponding to nucleotides 521–523 in the coding region of VEGF-B<sub>167</sub> cDNAs. The open reading frames of these mouse and human cDNA clones encoded polypeptides of 207 amino acid residues (Fig. 1A). Like VEGF-B<sub>167</sub>, the signal peptidase cleavage site is likely to occur between Ala<sup>21</sup> and Pro<sup>22</sup>. Thus the processed novel variants of VEGF-B, devoid of signal sequences, were 186 amino acid residues in length. We will refer to this isoform as VEGF-B<sub>186</sub>. Although the two variants of VEGF-B had an identical NH<sub>2</sub>-terminal domain of 115 amino acids, the COOH-terminal domains showed virtually no similarities. The eight invariant cysteine residues found in all PDGF/VEGF-like growth factors were within the common NH<sub>2</sub>-terminal domain. The COOH-terminal domains of mouse and human VEGF-B<sub>186</sub> displayed ~85% identity at amino acid level, which was similar to the identity displayed by the COOH termini of mouse and human VEGF-B<sub>167</sub> (~84%) (3). Data base searches revealed no significant amino acid sequence similarity between the COOH terminus of VEGF-B<sub>186</sub> and other sequenced proteins. In contrast, the COOH terminus of VEGF-B<sub>167</sub> is similar to that of VEGF (3). No consensus sites for N-linked glycrosylation (amino acid residues NX(S/T)) were found in the COOH-terminal domain of VEGF-B<sub>186</sub> and, as we have reported previously, no such sites are present in the common NH<sub>2</sub>-terminal domain (3). The COOH-terminal domains in mouse and human VEGF-B<sub>186</sub> were rich in conserved proline, serine, and threonine amino acid residues.

The divergence of the COOH-terminal amino acid sequences found in the two VEGF-B isoforms is reflected in their different biochemical characteristics. The COOH-terminal domain of VEGF-B<sub>186</sub> is weakly basic (net charge +5) and has a long stretch of hydrophobic amino acid residues in its COOH terminus (Fig. 1B). However, the hydrophobic tail in VEGF-B<sub>186</sub> does not serve as a transmembrane domain since this isoform is secreted from cells (see below). In comparison, the COOH-terminal domain of VEGF-B<sub>167</sub> is hydrophilic and strongly basic (net charge +13) and binds heparin (3). Thus, despite of their identical NH<sub>2</sub>-terminal domains, the two isoforms of VEGF-B have markedly different biochemical properties.

Exon-Intron Structures of Mouse and Human VEGF-B Genes—To investigate the possibility that the VEGF-B isoforms were generated by alternative splicing, the structures of the corresponding mouse and human genes were determined. The coding parts of the mouse and human VEGF-B genes were found to span ~4 kilobases of DNA, and both genes are divided into seven coding exons ranging from 19 bp (E7) to 236 bp in length (E6). The introns in both genes varied from 161 bp to ~2.6 kilobases (for a schematic outline, see Fig. 2). The nucleotide sequences of the exon-intron junctions of both genes are shown in Fig. 3. The length of each exon and the locations of the splice junctions in the mouse and human genes were identical, and all splice donor and acceptor sites followed the canonical
GT/AG rules (31). The only notable differences between the mouse and the human genes were the lengths of introns 1 and 4, which were longer in the human gene, and intron 6, which was longer in the mouse gene. All exon-intron boundaries were found to be well conserved between VEGF-B and VEGF, but the introns of the VEGF-B genes were generally smaller than the introns of the VEGF gene (18–20).

The 300-bp intron after exon 5 in VEGF-B differs from the human gene for VEGF, which is 3 kilobases in length and contains an alternatively spliced exon found in VEGF189 and VEGF206 (18, 19). When this intron in VEGF-B was analyzed more carefully, no exon corresponding to the sixth exon of human VEGF could be identified. Instead, the 3' ends of this intron and the following exon were found to be identical with corresponding sequences of the cDNA clones encoding mouse and human VEGF-B186. We conclude that the VEGF-B186 mRNA is formed by use of an alternative splice acceptor site during splicing, resulting in an insertion of a 101-bp intron sequence into these mRNAs.

VEGF-B167 and VEGF-B186 Are the Major Isoforms of VEGF-B—To determine whether the two VEGF-B splice isoforms exhibit a differential tissue distribution and whether additional isoforms exist, RT-PCR analysis was carried out using total RNA extracted from mouse brain, heart, liver, and kidney and from human embryonic heart and skeletal muscle. The cDNA sequences were amplified using two pairs of specific primers covering exons 4 to 6B or 7 and exons 3 or 4 to 7 in the mouse and human VEGF-B genes, respectively. Analysis of amplified PCR products from mouse tissues by agarose gel electrophoresis showed two major bands from each pair of primers used in the reactions. The lengths of these bands were 172 and 273 bp (using primers mp1 and mp2; see "Experimental Procedures") for the identity of the primers), and 223 and 324 bp in length (using primers mp1 and mp3, respectively) (Fig. 4A). Similar analysis of RNA from human fetal heart and skeletal muscle using two pairs of primers resulted in the amplification of bands of 223 and 324 bp (using primers hp1 and hp3) and 329 and 430 bp (using primers hp2 and hp3), respectively (data not shown). The amplified bands thus corresponded to the mRNAs for VEGF-B167 and VEGF-B186. Furthermore, amplification of mRNAs from different tissues yielded the two bands with roughly similar intensity, suggesting that the two isoforms were expressed at approximately equal levels in the tissues examined.

To verify the identity of the amplified products from mouse tissues, the PCR-amplified DNA from the primer combination mp1 and mp3 was transferred to a filter and probed with specific oligonucleotide probes for exons 6A and 6B. The autoradiograms showed that an exon 6A-specific probe hybridized with the 324-bp band, whereas an exon 6B-specific probe hybridized with both the 223- and 324-bp bands. Similarly, nucleotide sequence analysis of the amplified bands from the two human embryonic tissues showed that these bands were generated from transcripts encoding VEGF-B167 and VEGF-B186. These results confirmed that the two isoforms of VEGF-B are generated by alternative usage of the exon 6 splice acceptor sites and that both major amplified PCR products correspond to those predicted from the sequences of VEGF-B167 and VEGF-B186 isoforms (Fig. 4B).

Northern Blot Analysis of Transcripts Encoding VEGF-B186—The expression of VEGF-B186 transcripts in different tissues was investigated by Northern blotting and hybridization using a probe specific for the 101-bp insertion in VEGF-B186 mRNA (for details, see "Experimental Procedures"). The probe hybridized to VEGF-B186 cDNA but not to VEGF-B167 cDNA (Fig. 5A). The 1.4-kilobase mouse and human VEGF-B186 transcripts were expressed in several tissues in a pattern almost identical with that reported previously for both VEGF-B mRNAs (Fig. 5B) (3). These data support the conclusion from the RT-PCR analysis that expression of the VEGF-B isoforms is not tissue-specific.

Expression of VEGF-B186 in Transfected COS-1 Cells—Some biochemical properties of mouse VEGF-B186 were examined in COS-1 cells transfected with an expression vector. Immunoprecipitation and SDS-PAGE analysis of metabolically labeled proteins from transfected cells showed that cell-associated VEGF-B186 migrated as a 25-kDa polypeptide under reducing conditions (Fig. 6A). In contrast, VEGF-B186 present in the medium of transfected cells migrated as a 32-kDa protein, suggesting that VEGF-B186 undergoes a covalent modification during its intracellular transport and secretion (see below). The corresponding molecules were not detected in cell lysates or media from mock-transfected cells. Including 100 μg/ml heparin during the 2-h chase period did not affect the release of
Mouse VEGF-B<sub>186</sub> was expressed in COS-1 cells by transient transfection. The cells were then metabolically labeled as indicated. Cell culture medium (M) and a detergent-solubilized cell lysate (L) were immunoprecipitated using an antipeptide antibody to VEGF-B, and precipitated material was subjected to SDS-PAGE analysis under reducing conditions. Panel A, VEGF-B<sub>186</sub> was expressed as a 25-kDa protein in the cell lysate, whereas in the medium the protein appeared as a 32-kDa protein. As a control, mock-transfected COS-1 cells were analyzed in parallel. Panel B, VEGF-B<sub>186</sub> was immunoprecipitated from the medium and from the detergent-solubilized cell lysate. Aliquots of the immunoprecipitated material from the medium as a heterogeneous 32-kDa protein.

The biosynthesis of VEGF-B<sub>186</sub> homodimers was examined by pulse-chase experiments. Transfected COS-1 cells were metabolically labeled for 30 min and then chased for up to 240 min. Immunoprecipitation and SDS-PAGE analysis of detergent-solubilized cell lysates and media showed that VEGF-B<sub>186</sub> undergoes two modifications that affect its migration in SDS-PAGE (Fig. 6C). Following the labeling period of 30 min, VEGF-B<sub>186</sub> migrated as a distinct band of 25 kDa. After 30 min of chase a second diffuse band of around 26 kDa appeared. During longer chase periods the distinct 25-kDa band disappeared, whereas the diffuse 26-kDa band remained in the cell lysate for up to 240 min of chase. The secreted 32-kDa form of VEGF-B<sub>186</sub> appeared in the medium after 60 min of chase and accumulated in the medium at the highest concentration after the 240-min chase period. The 32-kDa form of VEGF-B<sub>186</sub> appeared heterogeneous in size. These results suggest that the intracellular 25-kDa protein corresponds to native unprocessed VEGF-B<sub>186</sub>, whereas the diffuse 26-kDa band corresponds to the protein with the first added O-linked glycan moiety, normally N-acetylgalactosamine. The addition of further glycans, including the terminal sialic acid moieties, subsequently generates the secreted 32-kDa mature VEGF-B<sub>186</sub>. The secretion of VEGF-B<sub>186</sub> was slow as the first detectable signals in the medium appeared after a 60-min chase.

VEGF-B<sub>186</sub> forms disulfide-linked homodimers and heterodimers with VEGF—We have shown previously that VEGF-B and VEGF are coexpressed in many tissues and that VEGF-B<sub>186</sub>-VEGF<sub>165</sub> heterodimers are formed readily when coexpressed in transfected cells (3). To examine whether also VEGF-B<sub>186</sub> could form heterodimers with VEGF<sub>165</sub>, COS-1 cells were transfected with the appropriate expression vectors, either alone or in combination. Metabolically labeled proteins present in the media and in detergent-solubilized cell lysates were subjected to immunoprecipitations using antisera to VEGF-B and VEGF and analyzed by SDS-PAGE. Analysis of media from transfected cells separately expressing VEGF-B<sub>186</sub> and VEGF showed that the two different antisera used were specific, with no detectable cross-reactivity (Fig. 7A). Immunoprecipitation and SDS-PAGE analysis of media and cell lysates from cells coexpressing VEGF-B<sub>186</sub> and VEGF showed intracellular and secreted heterodimers (Fig. 7B). When performing the SDS-PAGE analysis under nonreducing conditions, the secreted VEGF-B<sub>186</sub> homodimers migrated as a 60-kDa protein, VEGF-B<sub>186</sub>/VEGF heterodimers migrated as a 49-kDa protein.
In this work we have characterized a second isoform of VEGF-B encoding 186 amino acid residues, VEGF-B\textsubscript{186}. The novel isoform differs from the previously identified isoform, VEGF-B\textsubscript{167}, in a number of important aspects. The highly basic and cysteine-rich COOH-terminal heparin binding domain found in VEGF-B\textsubscript{167} is not present in VEGF-B\textsubscript{186}. As a consequence of this, the longer isoform is secreted freely from cells and does not remain bound to cellular or pericellular heparan sulfate proteoglycans. The COOH-terminal domain in VEGF-B\textsubscript{186} is rich in proline, serine, and threonine residues, many of which are conserved between human and mouse. It appears likely that this domain adopts an extended coil structure, whereas the COOH-terminal domain in the shorter isoform may be globular due to extensive intramolecular disulfide bonding.

The general features of mouse and human VEGF-B genes were identical with each other with the exception that the length of introns 1, 4, and 6 differed. Detailed analysis of both genes showed that the 167- and 186-amino acid isoforms of VEGF-B arise by alternative splicing from the same reading frame and have common COOH-terminal amino acid sequences. Thus, the isoforms of VEGF-B, VEGF, and PlGF are translated from the same reading frame and have common COOH-terminal domain in VEGF-B\textsubscript{167}. This suggests that both forms have distinct but important functional roles in VEGF-B physiology.

The exon-intron organization of the VEGF-B genes resembles those of VEGF and PIGF. For example, the common NH\textsubscript{2}-terminal domains of the different splice variants of these growth factors are encoded by exons 1–5, and the eight invariant cysteine residues are encoded by exons 3 and 4. The patterns of alternative splicing generating the different isoforms of VEGF-B, VEGF, and PIGF are similar, although the alternatively spliced exons in VEGF and PIGF are translated from the same reading frame and have common COOH-terminal amino acid sequences. Thus, the isoforms of VEGF-B appear to be generated by an exceptional mechanism involving a single set of alternative splicing events in the basic domain.

Secreted VEGF-B\textsubscript{186} was found to be modified by \textit{O}-linked glycosylation, presumably in its unique COOH-terminal domain, whereas no such modification can be expected in the shorter isoform (3). The addition of \textit{O}-linked glycans may serve to compensate for the hydrophobic character of the VEGF-B\textsubscript{186} COOH terminus and make the secreted protein more soluble in aqueous media. Among growth factors, \textit{O}-linked glycosylation is uncommon. It has been reported to occur in heregulins (32) and in a precursor of insulin-like growth factor II (33). In contrast, N-linked glycosylation of growth factors is observed frequently. Within the PDGF/VEGF family of growth factors,
all known isoforms, except VEGF-B\textsubscript{167}, contain N-linked carbohydrates. Thus, VEGF-B\textsubscript{186} represents the first isoform within this family which has O-linked glycans.

It was shown recently that a novel class of VEGF receptors, specifically interacting with sequences encoded by exon 7 of VEGF\textsubscript{165}, were expressed by some tumor cell lines (34). In this way, it appears likely also that the VEGF-B\textsubscript{167} isoform may interact with this novel class of receptors. This observation is particularly interesting since the corresponding exon 6B in VEGF-B\textsubscript{167} is highly homologous with exon 7 in VEGF, containing seven conserved cysteine and several conserved basic amino acid residues. The overall amino acid sequence identity between these two domains in VEGF and VEGF-B is around 50\% (3). It appears likely also that the VEGF-B\textsubscript{167} isoform may interact with this novel class of receptors for the heparin-binding portion of VEGF. Conceivably, alternative splicing, generating isoforms with distinct COOH-terminal domains, could regulate specificity for such receptors as it regulates the heparin binding properties of VEGF-B. However, the common NH\textsubscript{2}-terminal PDGF-like domain would be able to interact independently with VEGF receptor-like tyrosine kinases.

The ability of the two VEGF-B isoforms to form homodimers and to heterodimerize with VEGF, and perhaps with other similar growth factors, will increase the complexity of the cellular signals mediated via the various combinations of homo- and heterodimeric growth factors. This calls for a reevaluation of the functional role(s) of VEGF in vasculogenesis, angiogenesis, and vascular permeability. To sort out the specific and redundant signals for each of these growth factors in the normal physiology of endothelial cells during embryonic development and in adults, targeted mutations in each of the growth factors genes have to be generated and analyzed. With respect to VEGF-B, the characterization of the mouse VEGF-B gene, presented in this work, provides a basis for embarking into such studies.

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Note Added in Proof—Recently two papers by Grimmond et al. (Grimmond, S., Lagercrantz, J., Drinkwater, C., Silins, G., Townsend, S., Pollock, P., Gotley, D., Carson, E., Rakar, S., Nordenskjöld, M., Ward, L., Hayward, N., and Weber, C. (1996) Genome Res. 6, 124–131) and Townsend et al. (Townsend, S., Lagercrantz, J., Grimmond, S., Silins, G., Nordenskjöld, M., Weder, G., and Hayward, N. (1996) Biochem. Biophys. Res. Commun. 220, 922–928) have appeared, describing the human and murine VEGF-related gene (VRF), identical to VEGF-B.

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