Biosynthesis of Vascular Endothelial Growth Factor-D Involves Proteolytic Processing Which Generates Non-covalent Homodimers*

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Vascular endothelial growth factor-D (VEGF-D) binds and activates the endothelial cell tyrosine kinase receptors VEGF receptor-2 (VEGFR-2) and VEGF receptor-3 (VEGFR-3), is mitogenic for endothelial cells, and shares structural homology and receptor specificity with VEGF-C. The primary translation product of VEGF-D has long N- and C-terminal polypeptide extensions in addition to a central VEGF homology domain (VHD). The VHD of VEGF-D is sufficient to bind and activate VEGF-2 and VEGF-3. Here we report that VEGF-D is proteolytically processed to release the VHD. Studies in 293EBNA cells demonstrated that VEGF-D undergoes N- and C-terminal cleavage events to produce numerous secreted polypeptides including a fully processed form of Mₙ ~21,000 consisting only of the VHD, which is predominantly a non-covalent dimer. Biosensor analysis demonstrated that the VHD has ~290- and ~40-fold greater affinity for VEGF-2 and VEGF-3, respectively, compared with unprocessed VEGF-D. In situ hybridization demonstrated that embryonic lung is a major site of expression of the VEGF-D gene. Processed forms of VEGF-D were detected in embryonic lung indicating that VEGF-D is proteolytically processed in vivo.

Vascular endothelial growth factor-D (VEGF-D) was initially described in the mouse as a c-fos-induced growth factor (FIGF) capable of inducing mitogenesis of fibroblasts (1). It has since been identified in the human by homology cloning (2, 3) and designated VEGF-D based on its structural similarity to the VEGF family of growth factors, which include VEGF, VEGF-B, VEGF-C, placenta growth factor (PIGF), and viral VEGF proteins (reviewed in Refs. 4–6). These growth factors are secreted homodimeric glycoproteins, which contain a cystine knot motif that is essential for establishing the tertiary structure of the subunits (7). VEGF family members are involved in regulating the formation of blood vessels and lymphatic vessels within the developing embryo and adult, and in pathological situations such as tumorigenesis (reviewed in Refs. 4–6).

The VEGF family of ligands exert their effects on endothelial cells by binding to at least three endothelial cell-specific receptor tyrosine kinases designated VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR), and VEGFR-3 (Flt-4) (reviewed in Refs. 4–6). All three are broadly expressed on endothelial cells throughout embryonic development (8, 9), but, as embryogenesis proceeds, VEGF-3 becomes restricted to venous endothelial cells and then to endothelial cells of the lymphatic vessels (10). VEGF binds to VEGFR-1 and VEGFR-2 (9, 11, 12), whereas VEGF-B and PIGF bind only to VEGFR-1 (13, 14). In contrast, VEGF-C and VEGF-D bind VEGFR-2 and VEGFR-3 (3, 15). Two viral VEGFs from the NZ2 and NZ7 strains of orf virus bind to VEGFR-2 but not to VEGFR-1 or VEGFR-3 (16, 17). In addition to these three receptors, a non-tyrosine kinase receptor, neuropilin-1 (NP-1), was recently shown to bind VEGF₁₆₅ (18) and PIGF-2 (19). NP-1 enhances the binding of VEGF₁₆₅ to VEGFR-2 and its chemotactic and mitogenic responses (18). NP-1 also binds the viral VEGF from the NZ2 strain of orf virus (17). VEGFR-1, VEGFR-2, and VEGFR-3 are all critical for embryonic vascular development as mutant mice deficient in each of these receptors die during embryogenesis due to profound abnormalities of the vascular system (20–22).

From a structural viewpoint, VEGF-D is most closely related to VEGF-C. Indeed, the similarities in overall structure and receptor binding indicate that VEGF-D and VEGF-C form a subfamily within the vascular endothelial growth factors. The primary translation products of these two growth factors consist of a central VEGF homology domain (VHD), encompassing the cystine knot motif, and of N- and C-terminal polypeptide extensions that are not present in other VEGF family members (3, 15). The VHDs of VEGF-C and VEGF-D share 61% amino acid sequence identity (3). VEGF-C is lymphangiogenic (23, 24) and was recently shown to promote angiogenesis in an ischemic hindlimb model (25) and in mouse cornea (26). VEGF-C is initially synthesized as a prepropeptide, which is proteolytically processed to cleave off first the C-terminal polypeptide extension and then the N-terminal extension thereby yielding a mature, secreted form consisting only of the VHD (27). The degree of processing of VEGF-C serves to modulate the receptor specificity of the protein, e.g. the fully processed form binds VEGFR-2 and VEGFR-3 whereas partially processed forms bind only VEGF-3. Likewise, we have shown previously that a recombinant form of VEGF-D, consisting only of the VHD, is capable of binding and activating both VEGFR-2 and VEGFR-3...
(3). However, it was not known if VEGF-D is proteolytically processed to give rise to a form consisting only of the VHD.

In this study we demonstrate that VEGF-D is proteolytically processed to generate the bioactive VHD region. The fully processed form of VEGF-D consists only of the VHD and exists predominantly in the form of a non-covalent dimer. This material has greatly increased affinity for VEGFR-2 and VEGFR-3 when compared with the full-length VEGF-D. In addition, analysis of the distribution of VEGF-D mRNA by in situ hybridization demonstrated that the VEGF-D gene is strongly expressed in lung during mouse embryonic development. We were able to identify processed forms of VEGF-D in embryonic mouse lung, indicating that VEGF-D protein is processed in vivo.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Culture Conditions**—293EBNA cells were maintained in DMEM containing 10% (v/v) fetal bovine serum, 50 mg ml−1 gentamicin, 50 μg ml−1 genetamicin (supplements) in a humidified atmosphere of 10% CO2. Ba/F3 cells transfected with the VEGF-D/Epo receptor chimera were maintained in DMEM containing 10% (v/v) fetal bovine serum, 50 mg ml−1 gentamicin, 50 μg ml−1 genetamicin, 10% (v/v) WEHI-3D conditioned medium (a source of interleukin-3), and 1 mg/ml G418 in a humidified atmosphere of 10% CO2.

**Antisera**—A polyclonal antiserum, designated A2, was raised in rabbits against a synthetic peptide comprising human VEGF-D residues 190–205 in the VHD (KCLPTAPRHPHTSHIR) (Fig. 1). The numbering used is as defined previously (3).

**Truncated Forms of VEGF-D**—The human VEGF-D cDNA used in this study has been described elsewhere (3). DNA fragments encoding various derivatives of VEGF-D were generated from this cDNA by PCR with Pfu DNA polymerase (Stratagene, La Jolla, CA) and were subsequently cloned into pEFBOS-S-FLAG or pEFBOS-I-FLAG expression vectors (kindly supplied by Clare MacFarlane, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) in order to generate VEGF-D polypeptides, which were tagged with the FLAG octapeptide (IBI/Kodak, New Haven, CT). The constructs were designed to encode the VHD with the N terminus (VEGF-DANAC-FLAG), the full-length VEGF-D with an N-terminal flag sequence (VEGF-D-FULL-N-FLAG), the full-length VEGF-D with a C-terminal flag sequence (VEGF-D-FULL-C-FLAG), and a derivative with the C-terminal polypeptide extension removed and replaced with the FLAG peptide (VEGF-D-C-FLAG) (see Fig. 1). The construct for VEGF-DANAC-FLAG, previously designated as VEGF-DANAC, has been described in detail elsewhere (3). In the construct for VEGF-D-FULL-N-FLAG, DNA encoding the VEGF-D signal sequence for protein secretion had been deleted and substituted with DNA encoding the IL-3 signal sequence, followed by the FLAG octapeptide sequence and two amino acids (Thr-Arg) immediately after the initiation codon of VEGF-D. This construct also encoded the amino acids Ala-Arg-Gln, followed by the FLAG peptide sequence at the C terminus of the protein. The construct for VEGF-DANAC-FLAG encoded an N-terminal region identical to that of VEGF-D-FULL-C-FLAG, but the region encoding the C-terminal extension of VEGF-D (i.e. C-terminal to the VHD) had been deleted and replaced with DNA encoding the amino acids N-T-R-Q followed by an in-frame flag peptide, the two residues Thr-Arg, and two stop codons. Thus the sequence encoded immediately after Ile at position 212 of VEGF-D was N-T-R-Q-D-Y-K-D-D-K-T-R-STOP-STOP. Plasmid constructs were extensively sequenced to ensure no unwanted mutations were introduced. The expression cassettes were excised from all of the above plasmids with XbaI and inserted at the XbaI site of the expression vector pAPEX-3 (kindly supplied by Steve Squinto, Alexion Pharmaceuticals, CT) for transfection into 293EBNA cells.

**Transfections**—293EBNA cells were transfected using the CaPO4 method or with FuGene according to the manufacturer’s instructions (Roche Molecular Biochemicals, Mannheim, Germany). Colonies were selected in 100 μg/ml hygromycin in supplemented DMEM. Expressing clones were identified by immunoprecipitation of biosynthetically labeled conditioned medium using M2 gel and analysis by SDS-PAGE.

**SDS-PAGE and Western Blot Analysis**—Samples containing purified VEGF-D derivatives and lysates prepared from cells in culture and from the lungs of mouse embryos essentially as described elsewhere (28) were combined 1:1 with 2× SDS-PAGE sample buffer, boiled, and resolved by SDS-PAGE (29). For reduction, samples were either treated with 2% β-mercaptoethanol or in some instances with dithiothreitol immediately before electrophoresis. Supernatants were treated with 200 mM IAA in 20 mM Tris-HCl, pH 8, for 2 h at 22 °C prior to analysis by SDS-PAGE under reducing or non-reducing conditions. For Western blotting, the proteins were transferred to membrane and probed with M2 according to the manufacturer’s instructions or probed with anti-rabbit Ig-horseradish peroxidase (Bio-Rad) and developed with ECL, Amersham Pharmacia Biotech (Amersham, Bucks, UK).

**Metabolic Labeling and Pulse-Chase Experiments**—293EBNA cells expressing VEGF-D-FULL-N-FLAG were grown to 50% confluence, washed, and incubated in medium deficient in Cys-/Met- for 30 min. Cells were then labeled for 30 min in medium containing [35S]Cys/Met at 0.25 μCi/ml. After this period the labeled cells were chased in cold medium containing 15 mg/ml cysteine and 15 mg/ml methionine for 0, 15, 30, 60, 120, 360, or 1440 min (24 h). After these time periods, the supernatants were removed from the flasks and the cellular monolayers washed twice with cold PBS. The monolayers were then lysed according to previously described methods (28). The supernatants and cleared lysates were then immunoprecipitated with the A2 VEGF-D-specific antiserum and protein A-Sepharose beads (Amersham Pharmacia Biotech, Abingdon, UK) for 2 h at 4 °C. Beads were washed six times, boiled in 2× SDS-PAGE sample buffer, and analyzed by SDS-PAGE.

**Protein Purification, N-terminal Amino Acid Sequencing, and Size Exclusion Chromatography**—VEGF-D derivatives were purified from the conditioned medium of stably transfected 293EBNA cells by affinity chromatography on M2 (anti-FLAG) gel (IBI/Kodak, New Haven, CT), with elution using the FLAG peptide, according to the manufacturer. The FLAG peptide was removed using a centrifugal concentrator (Amicon, Beverly, MA). In some instances proteins were transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA) and probed with the anti-FLAG antiserum according to the manufacturer’s instructions (Sigma Aldrich Pty Ltd, Australia).

**Bioassay to Assess Capacity of Ligands to Bind to Cross-link VEGFR-2**—A bioassay was established in which Ba/F3 cells (IL-3-dependent) were stably transfected with a chimeric molecule containing the extracellular domain of mouse VEGFR-2 and the transmembrane and cytoplasmic domains of the mouse erythropoietin receptor (EpoR). Expression of the VEGF-EpoR chimeric receptor allows the cells to survive and proliferate in the presence of ligands for VEGF-R2; in the absence of IL-3. Cells expressing the VEGF-EpoR chimeric receptor (VEGF-2 bioassay cells) were washed three times in PBS, and once in medium lacking the 10% WEHI-3D conditioned medium supplement to remove residual IL-3. Cells (106) were aliquoted into microwell plates (Nunc, Denmark) containing dilutions of either WEHI-3D conditioned medium (as source of IL-3) or VEGF-DANAC-FLAG, cultured for 72 h and viable cells were then counted.

**Receptor Binding Assays**—The binding of VEGF-D derivatives to soluble forms of VEGF receptors was assessed using Ig fusion proteins consisting of the extracellular domains of human VEGF-R2 (VEGF-R2-Ig, Y. Gunji, Harkart Institute, Helsinki, Finland) or VEGF-R3 (VEGF-R3-Ig, K. Fujisola, Biotechnology Institute, Helsinki, Finland) and the Fe portion of human IgG. 293EBNA cells expressing VEGF-D derivatives were labeled with [35S]Cys/Met for 4 h and the conditioned medium was immunoprecipitated with the Ig fusion proteins, eluted in 2× SDS-PAGE sample buffer, boiled, and resolved by SDS-PAGE as described previously (3).

**Nuclear Protein Analysis**—Purified extracellular domains of VEGF-R2 (mouse VEGF-R2-FLAG) and human VEGF-R3 (VEGF-R3-Ig) were coupled to the carboxymethylated dextran layer of a sensor chip using standard amine coupling chemistry for analysis of the binding kinetics.

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using a BIAcore 2000 optical biosensor (Biacore, Uppsala, Sweden) (31). The residual activated ester groups were blocked by treatment with 1 M ethanolamine hydrochloride, pH 8.5, followed by washing with 10 mM diethyilamine to remove non-covalently bound material. Samples for analysis were diluted in HBS running buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween 20). The integrity of the bound VEGFR-2 and VEGFR-3 was assessed by binding of purified VEGF and VEGF-DΔNΔC-FLAG. Data was analyzed using BIAevalu-ation 3.0 (BIACORE, Uppsala, Sweden) assuming a 1:1 Langmuirian model.

Chemical Cross-linking—In Vitro Transcription—In Vitro—In vitro transcription using a combination of 35S-UTP and 32P-CTP (1250 Ci/mmol). Prior to hybridization, tissue sections were pretreated with proteinase K (2–40 μg/ml, optimized for embryonic age) for 30 min at room temperature, post-fixed in 4% paraformaldehyde, and treated with 0.25% (v/v) acetic anhydride in 0.1M triethylammonium hydrochloride, pH 8.0, for 10 min. The sections were incubated with a standard hybridization buffer containing 6 × 10⁴ cpm/μL probe in humidified chambers at 52 °C for 16–18 h. For autoradiographic detection, the slides were exposed to Kodak NTB-2 nuclear emulsion at 4 °C for 3 weeks, developed in Kodak D-19, and counterstained with Weigert’s iron hematoxylin. The two non-overlapping antisense RNA probes were homologous to the regions of mouse VEGF-D cDNA encoding from amino acid residues 1–85 (probe A) and 199–317 (probe B) using the amino acid numbering as published elsewhere (1). In addition to encoding the N-terminal 85 amino acids of VEGF-D, probe A also contained 80 nucleotides of the 5’-untranslated region immediately upstream from the translation start codon. Specificity of hybridization was confirmed using sense RNA probes.

**RESULTS**

**VEGF-D Is Post-translationally Processed by Proteolytic Cleavage**—The observation that the VHD of VEGF-D is sufficient to bind and activate VEGFR-2 and VEGFR-3 suggested that the primary translation product of VEGF-D, which contains long N- and C-terminal extensions in addition to the VHD, may be post-translationally processed (3). In order to investigate this possibility four plasmid constructs, VEGF-D-FULL-N-FLAG, VEGF-D-FULL-C-FLAG, VEGF-DΔC-FLAG, and VEGF-DΔNΔC-FLAG (Fig. 1), were stably transfected into 293EBNA cells, a cell line that is capable of proteolytically processing VEGF-C (27). Analysis of the conditioned medium from 293EBNA cells expressing VEGF-D-FULL-N-FLAG by affinity purification with M2 gel (anti-FLAG) and SDS-PAGE allowed specific analysis of only those VEGF-D polypeptides containing the FLAG octapeptide or of derivatives bound covalently or non-covalently to the FLAG-tagged polypeptides (Fig. 1). Analysis of the purified proteins under reducing conditions by silver staining revealed a species of Mₛ ~53,000, the expected size of unprocessed VEGF-D, as well as polypeptides of ~31,000 and ~29,000 (Fig. 2A). This result is consistent with proteolytic cleavage events occurring near the C terminus of the VHD. According to such a model, the Mₛ ~53,000 polypeptide would represent unprocessed VEGF-D and the Mₛ ~31,000 polypeptide would consist of the N-terminal propeptide and the VHD (i.e. lacking the C-terminal propeptide). The expected size of a polypeptide consisting of the N-terminal propeptide and the VHD is indeed of Mₛ ~31,000 because the VHD, which is glycosylated, was shown previously to be of Mₛ ~21,000 (3) and the expected size of the FLAG-tagged N-terminal propeptide is of Mₛ ~10,000. If processing of VEGF-D involves cleavage at the N terminus as well as the C terminus of the VHD, cells expressing VEGF-D-FULL-N-FLAG should also produce a Mₛ ~10,000 FLAG-tagged polypeptide consisting only of the N-terminal extension. Although a Mₛ ~10,000 polypeptide was not detected among the VEGF-D derivatives secreted by these cells as assessed by silver staining (Fig. 2A), it was clearly detected by Western blot analysis of the same material using M2 antibody (Fig. 2B). The Mₛ ~29,000 polypeptide detected by silver staining was not detected in the same sample by Western blot with M2 antibody (Fig. 2B) or with A2 antiserum (specific for the VHD) (Fig. 2C) and therefore represented the C-terminal propeptide. This was confirmed by N-terminal amino acid sequencing of this polypeptide, which identified the N-terminal sequence as “SIQIPEED,” demonstrating that the C-terminal cleavage site in VEGF-D is located immediately after arginine 205 (“SIQIPEED”) (Fig. 1). The Mₛ of the C-terminal peptide is larger than predicted (~21,000–22,000; size is based on the predicted peptide backbone of 16,957 plus ~5,000 in N-linked glycosylation) under reducing conditions, which may be due to the high cysteine content of this region. It is most likely
that the 29-kDa C-terminal propeptide was present in the affinity-purified material from cells expressing VEGF-D-FULL-N-FLAG because of interchain disulfide bonds between the N- and C-terminal propeptides. This is apparent from silver staining of this material under non-reduced conditions (Fig. 2D), where the 29-kDa band is absent. The major forms detected under non-reduced conditions are of Mr 85,000 and 50,000, which are most likely derived from partially processed dimers of VEGF-D in which only one or both C-terminal cleavage events had occurred, respectively.

To further examine the possibility of proteolytic cleavage of VEGF-D near the N terminus of the VHD, proteins secreted by 293EBNA cells expressing VEGF-DΔC-FLAG were purified and analyzed as above. The construct for VEGF-DΔC-FLAG drives expression of a VEGF-D derivative in which the C-terminal propeptide has been deleted and replaced with FLAG (Fig. 1). Conditioned medium from these cells contained two FLAG-tagged polypeptides of 31 and 21 kDa (Fig. 2F). This result is consistent with the model that the N-terminal extension and the VHD, whereas the 21-kDa polypeptide would consist of the VHD alone. Consistent with this model were the findings that the both the 31- and 21-kDa bands were detected by Western blot analysis with M2 antibody (Fig. 2G) and with A2 antiserum (data not shown). The identity of the 21-kDa polypeptide was confirmed by N-terminal amino acid sequencing. The N-terminal sequence of this polypeptide was heterogeneous. The predominant sequence, representing approximately 50% of the material, began as “FAATFY” and a minor sequence, representing 20% of the material, began with “KVIDEE.” Thus, as expected, the N terminus of the 21-kDa polypeptide is located at approximately the same position as the N terminus of the VHD. The major N-terminal cleavage site in VEGF-D is located immediately after arginine 88 (“R | FAATFY”) and the minor cleavage site is immediately after leucine 99 (“L | KVIDEE”). Thus, as expected, the N terminus of the 21-kDa polypeptide is located at approximately the same position as the N terminus of the VHD. The major N-terminal cleavage site in VEGF-D is located immediately after arginine 88 (“R | FAATFY”) and the minor cleavage site is immediately after leucine 99 (“L | KVIDEE”).

**Fig. 2.** Analyses of VEGF-D derivatives secreted by 293EBNA cells expressing VEGF-D-FULL-N-FLAG (A, B, C, D, and E), VEGF-DΔC-FLAG (F and G), and VEGF-D-FULL-C-FLAG (H). Proteins were purified from conditioned cell media by affinity chromatography using M2 (anti-FLAG) antibody. After SDS-PAGE, proteins were analyzed by silver staining (A, D, and F) or by Western blot analysis with M2 antibody (B, E, and G) or with A2 antiserum (C). Panel H shows analysis of proteins immunoprecipitated with anti-FLAG gel from the medium of metabolically labeled 293EBNA cells expressing VEGF-D-FULL-C-FLAG. L and S in panel B indicate results after long and short exposures, respectively. Samples are indicated as to whether they are analyzed under reducing conditions or non-reducing conditions. The positions of molecular mass markers (in kDa) are shown to the right of panel D and to the left of all other panels. The positions of VEGF-D derivatives (with Mr in kDa) are marked by arrows. For immunoblots (IB), the antiserum used are shown below the gels.

**Kinetics of VEGF-D Biosynthesis**—Pulse-chase analysis was used to study the kinetics and the mechanism of VEGF-D biosynthesis. 293EBNA cells expressing VEGF-D-FULL-N-FLAG were pulsed with [35S]Cys/Met and then chased at various times (from 15 min to 24 h) with medium containing unlabeled Cys/Met. Proteins from cell lysates were immunoprecipitated with the VHD-specific A2 antiserum. SDS-PAGE analysis of the immunoprecipitated material under non-reducing conditions (Fig. 3B) demonstrated that VEGF-D exists in the cell predominantly as a 53-kDa polypeptide and as a less
abundant species of $M_w \sim 105,000$. The absence of lower molecular weight species within the reduced cellular components indicated that there is no proteolytic processing of VEGF-D in the cell (Fig. 3B). The $\sim 105$- and $\sim 53$-kDa bands from the non-reducing gel were excised and analyzed by SDS-PAGE under reducing conditions. The $\sim 105$-kDa band was converted exclusively to a $\sim 53$-kDa form when reduced, whereas the $\sim 53$-kDa band was unaffected (data not shown). This indicated that the $\sim 105$-kDa band was a disulfide-linked dimer of unprocessed VEGF-D, whereas the $\sim 53$-kDa band was unprocessed VEGF-D that was not disulfide-bonded to other polypeptides.

Immunoprecipitation with antiserum A2 of polypeptides from the medium of 293EBNA cells expressing VEGF-D-FULL-N-FLAG demonstrated that VEGF-D is rapidly secreted and detectable in cell medium within 15 min after the onset of translation of radioactive VEGF-D (Fig. 3B). The predominant species observed under non-reducing conditions were $\sim 21$, $\sim 30$, $\sim 50$, and $\sim 85$ kDa in size. A faint band of $\sim 75$ kDa was also observed. Identical bands were seen when proteins from 293EBNA cells expressing VEGF-D, which had not been FLAG-tagged, were immunoprecipitated with antiserum A2 (data not shown). These bands derived from VEGF-D-FULL-N-FLAG were excised from the non-reducing gel and analyzed by SDS-PAGE under reducing conditions. Migration of the $\sim 30$- and $\sim 21$-kDa bands was unaffected by reduction, indicating that the former was the polypeptide containing the VHD and N-terminal propeptide and the latter was the mature form consisting of the VHD (data not shown). Upon reduction the $\sim 50$-kDa band migrated predominantly at $\sim 53$ kDa, i.e., as unprocessed VEGF-D, and a small proportion was converted to bands of $\sim 31$, $\sim 29$, and $\sim 24$ kDa, consistent with a molecule in which the C-terminal propeptide was disulfide-bonded to the polypeptide containing the VHD and N-terminal propeptide. The $\sim 24$-kDa band may have arisen due to proteolytic processing near the C terminus of the C-terminal propeptide. The $\sim 75$- and $\sim 85$-kDa bands were excised from a non-reducing gel together and the mixture analyzed under reducing conditions. This gave rise to strong bands of $\sim 50$, $\sim 30$, and $29$ kDa, and a weak band of $\sim 40$ kDa (data not shown). Therefore, the $\sim 50$- and $\sim 85$-kDa polypeptides consisted of various forms of partially processed, disulfide-linked VEGF-D polypeptides.

In contrast to the specific bands detected from the medium of cells expressing VEGF-D-FULL-N-FLAG, no bands were detected by immunoprecipitation with the A2 antiserum from the medium of metabolically labeled 293 cells transfected with an expression construct for VEGF164 (Fig. 3A). Also, the use of antiserum to mouse VEGF for immunoprecipitation from the medium of cells expressing VEGF-D-FULL-N-FLAG after a cold chase for 24 h revealed no specific bands (Fig. 3A). These controls demonstrated that the A2 antiserum was specific for VEGF-D proteins.

**The Fully Processed Form of VEGF-D Is Predominantly a Non-covalent Dimer**—In general, VEGF family members exist as disulfide-bonded homodimers. However, the VHD of VEGF-C, which binds and activates VEGFR-2 and VEGFR-3, exists predominantly in the form of a non-covalent dimer (27). We previously demonstrated that the VHD of VEGF-D (VEGF-DΔNΔC-FLAG) also binds and activates VEGFR-2 and VEGFR-3 (3), although the quaternary structure of this polypeptide was unknown. Analysis of purified VEGF-DΔNΔC-FLAG by SDS-PAGE and Western blot revealed that, when reduced, this material migrates exclusively at $\sim 21$ kDa, whereas under non-reducing conditions the vast majority of material migrates at $\sim 22$ kDa and a small proportion migrates as a high molecular mass aggregated form (Fig. 4A, I). In addition, the prior treatment of VEGF-DΔNΔC with IAA did not alter the appearance of the bands on SDS-PAGE indicating that disulfide bond shuffling due to destabilization of the structure in SDS was not occurring (Fig. 4A, II). Therefore, the predominant form of the mature, secreted VHD of VEGF-D is not a disulfide-bonded dimer. In order to further analyze the form of secreted VEGF-DΔNΔC-FLAG, we determined if this polypeptide could be cross-linked in cell supernatants by the
Fig. 4. Analysis of VEGF-DaNAC-FLAG by SDS-PAGE, chemical cross-linking, and size exclusion chromatography. A, I, analysis of VEGF-DaNAC-FLAG under reduced (R) and non-reduced (NR) conditions. For reduction, VEGF-DaNAC-FLAG was treated with dithiothreitol, then acetylated. Samples were subjected to SDS-PAGE, transferred to membrane, probed with the anti-FLAG antibody, and signals detected by chemiluminescence. The positions of molecular mass markers (in kDa) are shown to the left and right of lane 1. Lanes 1 and 2 correspond to protein from peaks 1 and 2, respectively. The position of the VEGF-DaNAC-FLAG subunit is indicated to the left, and the positions of molecular mass markers (in kDa) are shown to the right.

B, analysis of biosynthetically labeled VEGF-DaNAC-FLAG by immunoprecipitation with anti-FLAG gel and treatment with or without 200 mM IAA at pH 8, followed by SDS-PAGE under reducing (asterisk) conditions. For reduction, VEGF-DaNAC-FLAG was treated with dithiothreitol, and IAA was added. Samples were subjected to SDS-PAGE, transferred to membrane, probed with the anti-FLAG antibody, and signals detected by chemiluminescence. The positions of molecular mass markers (in kDa) are shown to the right. The asterisk (*) marks aggregated material. B, analysis of biosynthetically labeled VEGF-DaNAC-FLAG by immunoprecipitation with anti-FLAG gel and treatment with or without 200 mM IAA at pH 8, followed by SDS-PAGE under reducing (R) or non-reducing (NR) conditions. B, for chemical cross-linking, 293EBNA cells expressing VEGF-DaNAC-FLAG were metabolically labeled with [35S]Cys/Met and secreted proteins were collected in medium lacking serum. Half of the sample was incubated with the chemical cross-linker DSS (+), whereas the other half was incubated in the absence of DSS (−). VEGF-DaNAC-FLAG was immunoprecipitated from the samples and analyzed by SDS-PAGE under reducing conditions as described under “Experimental Procedures.” For comparison, medium containing labeled mouse VEGF164 was treated in the same way and immunoprecipitated with VEGF-specific antibody.

C, size exclusion chromatography of affinity-purified VEGF-DaNAC-FLAG was carried out on a TSKG2000SW column. Eluted proteins were monitored spectrophotometrically at 215 nm. Apparent molecular masses (in kDa) for the two peaks are shown within each peak in brackets, below the peak number. Molecular standards are indicated above the trace. Fractions corresponding to each of the two peaks were pooled, concentrated, and analyzed by SDS-PAGE under reducing conditions by silver staining and Western blotting with anti-FLAG antibody (D). Lanes 1 and 2 correspond to protein from peaks 1 and 2, respectively. The position of the VEGF-DaNAC-FLAG subunit is indicated to the left, and the positions of molecular mass markers (in kDa) are shown to the right.

The capacities of the dimeric and monomeric forms of VEGF-DaNAC-FLAG to bind and cross-link the extracellular domain of VEGFR-2 were assessed using a Ba/F3 cell bioassay for VEGFR-2 binding (see “Experimental Procedures”). The samples of VEGF-DaNAC-FLAG dimer and monomer in peaks 1 and 2 from the size exclusion chromatography, respectively, were tested in the bioassay immediately after elution from the column. The samples were tested over 3 logs of dilutions to compare relative activities. When matched for protein concentration the VEGFR-2-binding/cross-linking activity of the monomer in peak 2 was approximately 4% of the dimer in peak 1. Therefore, the VEGF-DaNAC-FLAG non-covalent homodimer is much more bioactive than the monomer. One can thus conclude that the dimeric form of VEGF-DaNAC-FLAG oligomerizes VEGFR-2 extracellular domains far better than does the monomeric form.

Receptor Binding of VEGF-D Derivatives—In order to determine the effect of proteolytic processing on the receptor binding capability of VEGF-D, biosynthetically labeled proteins from 293EBNA cells expressing VEGF-D-FULL-N-FLAG were immunoprecipitated with Ig fusion proteins consisting of the extracellular domains of human VEGFR-2 or VEGFR-3 and the Fe portion of human IgG1 (Fig. 5A). Six VEGF-D derivatives of ~53, 44, 31, 29, 24, and 21 kDa were precipitated with VEGFR-3-Ig (Fig. 5A, lane 1). From our analyses of VEGF-D proteolytic processing, it could be predicted that the ~53-kDa band was unprocessed VEGF-D, the ~31-kDa band consisted of the N-terminal propeptide and the VHD, the ~29-kDa band was the C-terminal propeptide, and the ~21-kDa band was the mature form consisting only of the VHD. The ~44-kDa band probably represented a polypeptide consisting of the VHD and the C-terminal propeptide and the ~24-kDa band an alternatively
To determine the relative affinities of unprocessed VEGF-D (VEGF-D-FULL-N-FLAG) and the VHD of VEGF-D (VEGF-DΔNAC-FLAG) for VEGFR-2 and VEGFR-3, we analyzed the relative binding kinetics for these interactions by biosensor analysis using surface plasmon resonance detection (31) (Fig. 5B). The VEGF-D-FULL-N-FLAG had been purified by M2 chromatography to minimize the abundance of partially processed derivatives and was devoid of fully processed VHD. VEGFR-2 and VEGFR-3 were immobilized onto a carboxymethylated dextran surface using amine coupling as described previously (4856 and 6947 response units immobilized, respectively, corresponding to 4.8 and 6.9 ng/mm²). Binding curves were obtained by flowing VEGF-D-FULL-N-FLAG or VEGF-DΔNAC-FLAG over the surface (38–380 nM) at a flow rate of 5 μl/min (Fig. 5B). The binding constants (Fig. 5C) were obtained by analysis of the initial dissociation phase to obtain the k_d, which was then used to constrain a global analysis of the association region of the curves, assuming a 1:1 Langmuirian model. The affinity for the interaction between VEGF-DΔNAC-FLAG and VEGFR-2 is ~290-fold greater than that for the interaction with VEGF-D-FULL-N-FLAG. In the case of the interactions with VEGFR-3, the affinity for VEGF-DΔNAC-FLAG is ~40-fold greater than that for VEGF-D-FULL-N-FLAG. This appears to be mainly due to a significantly reduced on rate for VEGF-D-FULL-N-FLAG compared with VEGF-DΔNAC-FLAG. Therefore, proteolytic processing increases the affinity of VEGF-D for both receptors, but the increase is ~7-fold greater for the interaction with VEGFR-2 than for VEGFR-3. It is possible that the immobilized receptor domains on the biosensor chip are unable to dimerize in response to VEGF-D binding. Therefore, the affinities measured here may be less than those determined in a cell-based system. Affinity measurements on cells were avoided for these studies to eliminate potential processing of VEGF-D-FULL-N-FLAG during the analysis.

**Analysis of VEGF-D Gene Expression during Embryonic Development**—In order to identify source tissue for further analysis of VEGF-D protein, VEGF-D gene expression was studied in post-coital day 15.5 mouse embryos by *in situ* hybridization (Fig. 6). The strongest signal for VEGF-D mRNA was detected in the developing lung. This signal was restricted to the mesenchymal cells; the epithelial cells of the bronchi and bronchioltes were negative, as were the developing smooth muscle cells surrounding the bronchi. The endothelial cells of bronchial arteries were also negative. Identical results were obtained using two non-overlapping antisense RNA probes. Controls with sense RNA probes were negative in all tissues (data not shown).

**VEGF-D Processing in Vivo**—Preliminary analysis of the proteolytic processing of VEGF-D *in vivo* was carried out by Western blot analysis with VHD-specific A2 antisera to tissue lysates prepared from the lungs of post-coital day 15.5 mouse embryos. Two bands of ~21 and ~30 kDa were detected under reducing conditions with the A2 antisera, which were undetectable with preimmune serum (Fig. 7). As these bands both contain the VHD, it can be predicted, given the characterization of VEGF-D processing in 293EBNA cells, that the ~21-kDa band is the mature secreted VHD and the ~30-kDa band is the secreted polypeptide consisting of the VHD and the N-terminal propeptide. These results indicate that VEGF-D is processed *in vivo* in a similar fashion to the processing that occurs in the medium of 293EBNA cells. Nonetheless our data do not exclude the possibility that there are different proteases processing VEGF-D *in vivo* compared with the *in vitro* processing observed in the culture medium of 293EBNA cells.
Our studies of VEGF-D processing in 293EBNA cells suggest a model for VEGF-D biosynthesis and secretion (Fig. 8) that is analogous to VEGF-C (27) and has some similarities to the processing of the PDGF polypeptides (33, 34). According to this model, VEGF-D is produced as a prepropeptide of 53 kDa, which is rapidly secreted from the cell. Minimal processing occurs within the cytoplasm as intracellular proteolytically processed forms of VEGF-D were undetectable. Some of the unprocessed VEGF-D in the cell is monomeric, and some is in the form of a disulfide-bonded homodimer, which is 105 kDa under non-reducing conditions and is converted to 53 kDa upon reduction. The intersubunit disulfide bonds in this dimer do not involve the VHD because the mature form of VEGF-D, consisting only of the VHD, is predominantly a non-covalent homodimer. Therefore, the intersubunit disulfide bonds most likely form between the N- and C-terminal propeptides (see Fig. 8), which would ensure that the two VHDs in the dimeric complex are aligned in an anti-parallel fashion, as is the case for VEGF (35). Such disulfide bonding is feasible, given that the N-terminal propeptide contains one cysteine residue and the C-terminal propeptide contains 20. After or during secretion, VEGF-D can be proteolytically cleaved at the N and C termini of the VHD. Two N-terminal cleavage sites were identified that differ from, although they are less than eight amino acid residues from, those for VEGF-C (27). A unique C-terminal cleavage site was identified, which is in the same position as that for VEGF-C and is immediately C-terminal to two basic arginine residues (27). The relative abundance of various partially processed VEGF-D derivatives detected in cell culture indicated that cleavage at the C terminus of the VHD is more efficient than at the N terminus. Nevertheless, cleavage at the N terminus was efficient enough to generate significant quantities of the mature form of VEGF-D. Both mature VEGF-D and a form consisting of the VHD and the N-terminal propeptide were detected in embryonic mouse lung, indicating that proteolytic processing of VEGF-D occurs in vivo. However, the proteases responsible for processing VEGF-D in vivo may differ from those that cleave this growth factor in the medium of 293EBNA cells.

The synthesis of precursor peptides that are proteolytically processed is common among growth factor families. In the case of VEGF-C, proteolytic processing is very similar to that for VEGF-D (27). For the PDGF B-chain, proteolytic cleavage occurs at the N and C termini of the region that aligns in the primary structure with the VHDs of VEGF-D and VEGF-C. As for VEGF-D and VEGF-C, this gives rise to a mature form...
containing the cystine knot motif; however, proteolytic processing for PDGF-B is intracellular (33, 34). Our receptor binding data demonstrate that processing of VEGF-D is required to produce a growth factor that binds VEGFR-2 and VEGFR-3 with high affinity. The fully processed form of VEGF-D binds in vivo the receptor affinity, respectively, than does unprocessed VEGF-D. Therefore, proteolytic processing is likely to regulate VEGF-D bioactivity in vivo. As the increase in affinity for VEGFR-2 due to processing is 7-fold more than that for VEGFR-3, processing may, in effect, modulate receptor specificity in vivo as unprocessed VEGF-D at physiological concentrations may bind VEGF-3 but not VEGF-2. In this scenario, processing would be an absolute requirement for VEGFR-2 binding but not for VEGFR-3-binding. The identification of the protease(s) responsible for VEGF-D processing will be important for determining the biological context of the regulation of the receptor affinity and specificity of VEGF-D.

The absence of interchain disulfide bonds in the homodimers of VEGF-D and VEGF-C is surprising, given that the other members of the PDGF superfamily are covalent dimers. In VEGF, the interchain disulfide bonds are crucial for dimerization and bioactivity (36) but are not essential for the dimerization and mitogenicity of PDGF-BB (37, 38). The dimer interface of PDGF-BB is sufficient to substantially stabilize the dimer in the absence of disulfide bonds (39). It may be that alterations in amino acid sequence between VEGF and VEGF-D/VEGF-C allow the latter growth factors to form stable non-covalent dimers. The VHDs of both VEGF-D and VEGF-C contain, in addition to the eight cysteine residues conserved within the VHDs of all VEGF family members, an extra cysteine residue (amino acid 117 of human VEGF-D) located six amino acids C-terminal to the first conserved cysteine residue (3). It may be possible that this extra cysteine residue is involved in subverting the intersubunit disulfide bonds that are normally seen in VEGF family members other than VEGF-C and VEGF-D. Determination of the crystal structure of VEGF-D will allow analysis of the residues important for stabilization of the dimer.

That VEGF-D and VEGF-C constitute a subfamily of the PDGF/VEGF family is demonstrated by the similarities in: (i) proteolytic processing of these proteins, (ii) receptor specificities of the mature forms, and (iii) the non-covalent nature of the mature forms. Analysis of mice deficient in VEGF-D and VEGF-C will help to further define the biological functions of these growth factors.

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FIG. 8. Schematic representation of VEGF-D processing by 293EBNA cells. Two forms of unprocessed VEGF-D are secreted from the cell: a monomer (left side) and a disulfide-linked dimer (right side). The dimer is assumed to have an anti-parallel configuration based on the known structure of other VEGF family members (35). Arrows lead from the intracellular forms to the products of stepwise proteolytic processing, which give rise to a mature form that is predominantly a non-covalent dimer of the VHD. However, not all polypeptides become fully processed; therefore, unprocessed and partially processed forms are detected in cell culture supernatants. Dimeric derivatives in which N- or C-terminal cleavage has occurred in only one subunit exist, but, for simplicity, are not shown here. All combinations of subunits with no processing or partial processing can be envisaged. N-pro denotes the N-terminal propeptide; C-pro, the C-terminal propeptide; VHD, the VEGF homology domain; dotted lines, non-covalent interactions between domains; -S-S-, intersubunit disulfide bridges; -N-, the N termini of polypeptides; arrowheads, the approximate locations of proteolytic cleavage sites.
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