Extracellular Cleavage of the Vascular Endothelial Growth Factor 189-Amino Acid Form by Urokinase Is Required for Its Mitogenic Effect*

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Vascular endothelial growth factor (VEGF) mRNA results in three distinct molecular forms of 121 or 165 (V165) amino acids that are released in the conditioned medium of cultured cells and one longer isoform of 189 amino acids (V189) that remains cell-associated. V189 has been expressed in wild-type CHO-K1 cells and in glycosaminoglycan-deficient pgsA-745 Chinese hamster ovary (CHO) mutant cells. It could be released from CHO-K1 cell membranes by heparin or a synthetic peptide designed on the sequence encoded by exon 6 but was freely released from CHO mutant cells. In both cases, the immunoreactive V189 was mainly released as a 40-kDa cleaved form, provided that the serine protease urokinase, but not plasmin, was active. Recombinant V189 was purified from insect cells infected with a recombinant baculovirus as a nonmitogenic 50-kDa precursor that binds to the receptor Flt-1 but not to Flk-1. It could be matured by urokinase as a 38-kDa fragment able to bind to Flk-1 and to trigger cell proliferation. V165 and V189, however, could be cleaved by plasmin as 34-kDa fragments that exhibit a decreased mitogenic activity. These findings indicate that the carboxyl-terminal domain of V189 masks its binding domain to Flk-1.

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Alternative splicing of vascular endothelial growth factor (VEGF) mRNA results in three distinct molecular forms of 121 or 165 (V165) amino acids that are released in the conditioned medium of cultured cells and one longer isoform of 189 amino acids (V189) that remains cell-associated. V189 has been expressed in wild-type CHO-K1 cells and in glycosaminoglycan-deficient pgsA-745 Chinese hamster ovary (CHO) mutant cells. It could be released from CHO-K1 cell membranes by heparin or a synthetic peptide designed on the sequence encoded by exon 6 but was freely released from CHO mutant cells. In both cases, the immunoreactive V189 was mainly released as a 40-kDa cleaved form, provided that the serine protease urokinase, but not plasmin, was active. Recombinant V189 was purified from insect cells infected with a recombinant baculovirus as a nonmitogenic 50-kDa precursor that binds to the receptor Flt-1 but not to Flk-1. It could be matured by urokinase as a 38-kDa fragment able to bind to Flk-1 and to trigger cell proliferation. V165 and V189, however, could be cleaved by plasmin as 34-kDa fragments that exhibit a decreased mitogenic activity. These findings indicate that the carboxyl-terminal domain of V189 masks its binding domain to Flk-1.

Vascular endothelial growth factor (VEGF) is an angiogenic growth factor purified from the conditioned medium of a variety of cell types including the tumoral cell line AtT20 derived from mouse anterior pituitary (1), bovine folliculostellate cells (2), human monocytes (3), guinea pig tumor (4), and a rat glioma cell line (5). This secreted growth factor, also called vasculotropin (1) or vascular permeability factor (3), is a homodimer of 45 kDa sharing an overall homology of 18% with the B chain of platelet-derived growth factor. It stimulates in vitro not only the proliferation of vascular endothelial cells but also that of interleukin-2-dependent lymphocytes (6), retinal pigment epithelial cells (7), and hair dermal papilla cells (8). In vivo it induces angiogenesis (1) and vascular permeability (3, 4). Molecular cloning of human cDNA has shown that the factor is in fact composed of multiple species that are generated after alternative splicing of a single gene transcription product (9–12). The transcript encoding the 165-amino acid form is expected to generate the 45-kDa peptide following signal peptide cleavage (V165). A shorter transcript encodes V121, which presents a 44-amino acid deletion between positions 116 and 159 when compared with V165. A longer transcript encodes a 189-amino acid isoform (V189) with a 24-amino acid insertion at position 116. This 24-amino acid peptide corresponds to exon 6 of the gene. A fourth molecular species, V206, identical to V189 but containing 17 additional codons following the 24-codon insertion appears only to be expressed in embryonal tissue (11). The significance of these observations is not known in terms of bioactivity. Houck et al. (13) have suggested that, contrasting with V165, which is secreted and freely soluble in the tissue culture medium, the bioavailability of the secreted 165 and more specifically of the 189 forms appears to be regulated by binding to heparan sulfate proteoglycans in the extracellular matrix. This would provide a reserve of biologically active growth factor from which the V189 species could be released by heparin, heparan sulfate, and heparinase or through proteolysis following plasminogen activation. They also showed that both V165 and V189 were released from their bound states as 34-kDa dimeric species that were active as endothelial mitogens and as vascular permeability agents. Possible mechanisms for these biological activities would be the triggering of a protease cascade to digest the extracellular matrix and the activation of latent growth factors. Such a process has been described for transforming growth factor-β (14) and for pro-hepatocyte growth factor/scatter factor (HGF). It has been shown that the maturation of pro-HGF into a bioactive dimer takes place in the extracellular environment under the influence of the urokinase-type plasminogen activator (uPA) both in vitro and in vivo (15, 16) and that this process constitutes a crucial limiting step in the HGF signaling pathway after its secretion.

We studied the maturation process of V189 under the influence of plasmin or uPA, comparing Chinese hamster ovary cells (CHO-K1) and related glycosaminoglycan-deficient pgsA-745 mutant cells permanently expressing the 189 isoform. In agreement with the data from Houck et al. (13) we found that V189
was secreted but was entirely bound to the cell surface or the extracellular matrix of CHO-K1 cells. It could be released as 52- and 40-kDa fragments by heparin or a synthetic peptide designed on the sequence encoded by the exon 6. In contrast, we observed that V189 was directly released in the extracellular environment of heparan sulfate-defective cells as a biologically active 40-kDa fragment, suggesting that the 52-kDa precursor in such cell populations was readily accessible to a spontaneous maturation process from which it would be protected in CHO-K1 cells. V189 was expressed in insect cells and purified as 38- and 50-kDa proteins, which were compared with native V165 with respect to various biochemical and biological functions. Both forms bound to the VEGF receptor Flt-1, but the 50-kDa form could bind to the VEGF receptor Flk-1 and exert a mitogenic activity only after maturation by uPA or plasmin.

**EXPERIMENTAL PROCEDURES**

**Materials—**Ectodomain recombinant VEGF was from Bio-Rad, chromatography reagents were from Pharmacia Biotech, Inc., and Na2EDTA was from Amersham Corp. Heparin was obtained from Chony, and aprotinin, leupeptin, and goat anti-chicken immunoglobulins conjugated to fluorescein isothiocyanate were from Sigma. Anti-extracellular matrix capillary was from Stago, and anti-urokinase monoclonal antibody was from American Diagnostics. Cell culture reagents were from Life Technologies, Inc. Cell culture trays were from Costar. The clones pUC-121, pUC-165, and pUC-189 were obtained from Dr. J. Abraham (Scios-Nova, Mountain View, CA). Recombinant human V165 was produced from the conditioned medium of infected insect Sf9 cells using a recombinant baculovirus expression system as described by the manufacturer (CLONTECH). The purification procedure has already been described (1). Recombinant FGF2 was prepared in *Escherichia coli* as described (17).

**Expression and Purification of V189—**Recombinant V189 was produced in Sf9 insect cells using the recombinant baculovirus expression system. V189 expressing cells (10^6 cells) were extensively washed 3 days postinfection with phosphate-buffered saline supplemented with 5 μg/ml each of leupeptin, aprotinin, and pepstatin, extracted in 10 mM phosphate buffer, pH 7.2, containing 1 mM EDTA, 1% Triton X-100, and 0.1% sodium dodecyl sulfate supplemented with 2 mM NaCl and then adjusted to 0.2 mM NaCl and loaded on an SP-Sepharose cation exchange chromatography column, which had been equilibrated in the same buffer. After loading, the column was washed with the phosphate buffer adjusted to 0.4 mM NaCl, and V189 was eluted with 1 mM NaCl. This material was diluted twice, loaded on a heparin-Sepharose affinity chromatography column, and eluted by a 0.5–1.5 M NaCl gradient. V189 was measured in the fractions by an enzyme-linked immunosorbent assay as described below.

**Eukaryotic Expression Vectors and Transfection of CHO Cells—**CHO-K1, parental wild-type CHO cells, and pgsA-745 mutant CHO cells expressing xylanyltransferase deficiency (21) were routinely grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin, 0.25 μg/ml fungizone, and 2 mM L-glutamine. The cDNA fragment corresponding to V189 was subcloned into a baculovirus eukaryotic expression vector, pBR-EN, containing the Neo gene as a selective marker (22). In this construct, the VEGF cDNA is directly placed downstream of the cytomegalovirus promoter element as a first open reading frame, and the Neo gene is directly placed downstream of the internal entry of the encephalomyocarditis virus mRNA as the second open reading frame. A phage T7 RNA polymerase promoter is positioned downstream of the cytomegalovirus promoter immediately after the transcription start. Cells were transfected with the resulting expression plasmids or the control plasmid using a lipofection technique (Life Technologies, Inc.) as described by Brunette et al. (23). Stable integrants were selected using 500 μg/ml G418, and cloning was carried out by colony isolation using a Pasteur pipette. Expression of specific mRNAs was assessed by reverse transcriptase-polymerase chain reaction on reverse transcribed RNA using a T7 promoter oligonucleotide and a reverse oligonucleotide complementary to the 3’ end of the VEGF coding sequence. The conditioned media were prepared by exposing confluent cells to serum-free DMEM supplemented with antibiotics and 5 μg/ml insulin and 10 μg/ml transferrin either alone or supplemented with 50 μg/ml heparin, synthetic peptides, anti-uPA IgG, α2 antiplasmin, or 50 μg/ml leupeptin or aprotinin.

PsgA-745 CHO cells were also transfected with a mixture of 10 μg of pSV7-4 expression vectors carrying the Flt-1 and Flk-1 coding sequences (24, 25) and 1 μg of pS2V expression vector carrying the neomycin resistance gene with the Lipofectin method as described above. Transformants were screened by their ability to specifically bind iodinated VEGF.

**Growth Factor Bioassays—**Bovine endothelial cells cultured from adrenal cortex capillaries (ACE) were grown in DMEM supplemented with 10% calf serum, 2 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 0.25 μg/ml fungizone. Stock cultures received 1 mg of FGF2 every other day (26).

Subconfluent pSV-7d Flk-1 and pSV-7d Flt-1-transfected psgA-745 cells were rinsed with cold binding buffer (DMEM supplemented with 2 mg/ml gelatin and 20 mM HEPES, pH 7.4) and transferred to 4 °C, and incubated with various dilutions of unlabeled V165 or V189 in the presence of 5 ng/ml ^125I-V165. After 2 h, the cells were washed three times with cold binding buffer and lysed with 0.5 ml of 0.2 M NaOH. Solubilized material was counted in a γ counter. The non-specific binding was determined in the presence of 2 μg/ml of V165 and was less than 30%. In another set of experiments, ACE cells were incubated with 5 ng/ml of iodinated V165 or V189, either native or cleaved with plasmin or uPA, and treated as above.

For proliferation assays, ACE cells were seeded at 5000 cells/well in 12-well cluster plates in 1 ml of culture medium. Increasing concentrations of growth factors were added every other day, and cells were trypsinized and counted on day 4. Neither the treatments used to cleave V165 or V189 (plasmin, uPA) nor heparin affected ACE cell proliferation. At intermediate concentrations used, VEGF content was measured in conditioned media by a radioreceptor assay already described (18). Western blot analysis of the conditioned medium was performed by electrophoresis on 12% polyacrylamide gels in 0.1% SDS followed by transfer to nitrocellulose. After blocking with 5% nonfat milk in 50 mM Tris, pH 7.6, containing 0.1% Tween 20 (TBST), incubation was carried out with 2 μg/ml of anti-VEGF IgG, followed by a 1:10,000 dilution of anti-rabbit IgG conjugated with peroxidase. The antigen-antibody complex was detected by the ECL detection system (Amersham) according to the manufacturer’s instructions. Enzyme-linked immunosorbent assay was performed on 96-well dishes coated with 2 μg/ml immunopurified anti-V121 chicken Ig diluted in 50 mM carbonate buffer, pH 9.6, and further blocked with 10 μg/ml bovine serum albumin in the same buffer. Serial dilutions in TBST of V165 and V189 were incubated for 3 h at 37 °C, washed, and incubated an additional 1 h with 0.5 μg/ml of immunopurified anti Ex6P-IgG. Immuno-complexes were revealed with anti-rabbit IgG conjugated with peroxidase. The antigen-antibody complexes were detected by orthophenylenediamine substrate, and the optical density was read at 492 nm in a Dynatech Microlisa reader.

**Flow Cytometry—**Confluent V189- and control-transfected CHO K1 and pgsA-745 cells were rinsed twice with phosphate-buffered saline, 2% bovine serum albumin and further incubated 2 h at 37 °C in the presence or absence of 50 μg/ml of heparin or Ex6P. The cells were then resuspended for 1 h with 20 μg/ml purified chicken anti-VEGF antibody, rinsed, and further incubated with commercial goat anti-chicken immunoglobulins labeled with fluorescein isothiocyanate diluted 1:50 in Dulbecco’s phosphate-buffered saline. 35,000 cells were counted, and the fluorescence was analyzed with a Coulter Counter ELITE (excitation, 488 nm; emission, 522 nm). The addition of propidium iodide allowed the exclusion of nonviable cells.

**Cleavage by Serine Proteases—**V165 or V189 was mixed with 10
leupeptin. Cleavage of proteins was monitored by SDS-polyacrylamide gel electrophoresis after 48 h of incubation with 50 μg/ml heparin or Ex6P as indicated. The reaction was quenched, if desired, by the addition of 5 μg/ml of uPA in 200 μl of saline/bovine serum albumin with or without 50 μg/ml of heparin or Ex6P at 4 °C as indicated. The cell suspension was extensively washed and further incubated in the same buffer containing 20 μg/ml of affinity-purified chicken anti-V121 antibody, rinsed, and further incubated in a 1× dilution of isothiocyanate-labeled goat anti-chicken antibody. After washing, the cell suspension was injected in an ELITE flow cytometer and analyzed with a laser fluorimeter. The mean fluorescence of control transfected cells was below 0.7. Nontiable cells were excluded from the recording by the use of propidium iodide. The experiments were repeated three times with similar results.

RESULTS

CHO Cells Overexpressing Human V189 Release the Growth Factor in the Culture Medium as a Cleaved Product—Human V189 mRNA was expressed in K1 and mutant CHO cells as judged by reverse transcriptase-polymerase chain reaction using a 5′ oligonucleotide complementary to the distal sequence of VEGF (data not shown). No signal was detected in vector-transfected cells. Flow cytometry analysis showed that untransfected K1 cells exhibited a mean fluorescence of <0.7 arbitrary units, whereas that of their transfected counterparts rose to 10.2 (Fig. 1). Incubation of the cells for 3 h at 4 °C in the presence of heparin or Ex6P led to a significant reduction of the signal (1.8 and 1.1 arbitrary units, respectively). No VEGF-like activity was detected by radioreceptor assay in the conditioned medium alone, whereas heparin and Ex6P released similar amounts of VEGF (Table I). The peptide Ex6sP containing a shorter sequence encoded by exon 6 released almost as much VEGF (Table I). The peptide Ex6sP containing a shorter sequence encoded by exon 6 released almost as much VEGF (Table I). The peptide Ex6sP containing a shorter sequence encoded by exon 6 released almost as much VEGF (Table I). The peptide Ex6sP containing a shorter sequence encoded by exon 6 released almost as much VEGF (Table I). The peptide Ex6sP containing a shorter sequence encoded by exon 6 released almost as much VEGF (Table I). The peptide Ex6sP containing a shorter sequence encoded by exon 6 released almost as much VEGF (Table I). The peptide Ex6sP containing a shorter sequence encoded by exon 6 released almost as much VEGF (Table I). The peptide Ex6sP containing a shorter sequence encoded by exon 6 released almost as much VEGF (Table I). The peptide Ex6sP containing a shorter sequence encoded by exon 6 released almost as much VEGF (Table I). The peptide Ex6sP containing a shorter sequence encoded by exon 6 released almost as much VEGF (Table I). The peptide Ex6sP containing a shorter sequence encoded by exon 6 released almost as much VEGF (Table I). The peptide Ex6sP containing a shorter sequence encoded by exon 6 released almost as much VEGF (Table I). The peptide Ex6sP containing a shorter sequence encoded by exon 6 released almost as much VEGF (Table I). The peptide Ex6sP containing a shorter sequence encoded by exon 6 released almost as much VEGF (Table I). The peptide Ex6sP containing a shorter sequence encoded by exon 6 released almost as much VEGF (Table I).

Two distinct peaks corresponding to 2.2 and 32.1, respectively, were detected by flow cytometry analysis in V189-transfected pgsA-745 cells (Fig. 1). The signal corresponding to the peak displaying the lower fluorescence intensity was shifted to the negative range, whereas the second remained detectable upon heparin or Ex6P treatments. VEGF-like activity was not detected in the corresponding conditioned medium (Table I). Heparin, Ex6P, and Ex6sP released a significant amount of VEGF-like activity, whereas SP was almost ineffective. Western blot analysis of the proteins in the conditioned medium showed that V189 migrated as two molecular species of 52 and 40 kDa (Fig. 2). Interestingly, uPA inhibitors leupeptin and anti-uPA antibody prevented the release of V189 immunoreactivity, whereas plasmin inhibitors were inefficient. Parallel Western blots of cell-associated V189 showed that the less V189 was released, the more it remained cell-associated, confirming that protein synthesis was not affected by uPA or plasmin inhibitors. The addition of heparin to the cultures increased the total amount of immunoreactive V189 isoforms, regardless of plasmin inhibition (Fig. 2). In contrast, heparin induced the release of only the native 52-kDa form from urokinase-inhibited cells, confirming that V189 cleavage was not a prerequisite for cell release.

Recombinant V165 and V189 Are Differently Cleaved by Plasmin and Urokinase—To distinguish between the biological
properties of the cleaved and the native forms, V189 was expressed in Sf9 insect cells infected with a recombinant baculovirus carrying V189 cDNA. The cell lysate was subjected to cation exchange chromatography, and the V189 immunoreactivity eluted between 0.4 and 1.0 M NaCl was further purified by heparin affinity chromatography. V189 immunoreactivity was resolved in two molecular species eluted at 0.7 and 1.2 M NaCl whose molecular masses (38 and 50 kDa, respectively) were 2 kDa lower than those of the corresponding V189 isoforms observed in pgsA-745 CHO cells, probably due to different glycosylations occurring in mammalian and insect cells. The purified recombinant V189 50-kDa and V165 forms were then iodinated and subjected to serine protease cleavage and analyzed by autoradiography. No sign of self-activation of V189 or V165 could be detected. As shown in Fig. 3, plasmin generated an identical 34-kDa molecular species from both V165 and V189 (Pl-V189). The resulting peptides with an expected size of 12 kDa for V165 or 16 kDa for V189 were not visualized. uPA did not cleave V165 but generated a 38-kDa from V189 (uPA-V189). The purified 50- and 38-kDa V189 forms and the uPA-cleaved 38-kDa V189 were equally recognized in an enzyme-linked immunosorbent assay using IgG directed against Ex6P, whereas Pl-V189 and V165 were not (Fig. 4). V189 Maturation by Urokinase or Plasmin Is Required for Binding to Flt-1-expressing Cells—We examined the effects of mild plasmin or uPA cleavage of 50-kDa V189 by a radioreceptor assay using pgsA-745 CHO cells expressing constitutively Flt-1 or Flk-1 as target cells. Various concentrations of V165, 38- and 50-kDa V189, and the 34- and 38-kDa fragments generated by plasmin or uPA were tested for their ability to compete with the cell binding of iodinated V165 in the presence of 5 μg/ml of leupeptin to inhibit the putative cell maturation of V189. V165 and 38- and 50-kDa V189 forms competed almost equally to unlabeled V165. Radioiodinated V165 binding to Flt-1-expressing cells was halff-maximally displaced by 47, 92, and 75 pM V165, 38-kDa V189, and 50-kDa V189, respectively (Fig. 5A). uPA cleavage did not affect the competing activity of V189 (IC50 = 68 pM), whereas plasmin reduced the competing activity 4-fold (IC50 = 281 pM). Surprisingly, 50-kDa V189 was not able to displace iodinated V165 from Flk-1-expressing cells (IC50 = 316 and 289 pM as compared with 184 pM for V165). In contrast, Pl-V189 was less potent than uPA-V189 (IC50 = 1,052 pM).

V189 Maturation by Urokinase or Plasmin Is Required for Mitogenicity—To determine if V189 maturation could occur at the target cell level, bovine adrenal cortex capillary endothelial cells were incubated with 5 ng/ml of various preparations of

FIG. 3. Cleavage of V189 and V165 by urokinase and plasmin. 1 ng of iodinated recombinant V165 or V189 were subjected or not subjected (CON) to urokinase (uPA) or plasmin (Pl) treatment for 16 h at 37 °C, and the reactions were stopped by the addition of denaturing buffer. The products of the reaction were separated on a 15% SDS-polyacrylamide gel electrophoresis followed by autoradiography.

FIG. 4. Effects of V189 proteolytic processing on its immunoreactivity toward anti-Ex6P IgG. Various concentrations of V165 (●), 50-kDa V189 (■), 38-kDa V189 (▲), uPA-V189 (△), or Pl-V189 (▲) were coated in microtiter plates in 50 mM carbonate buffer, pH 9.6. 0.5 μg/ml of immunopurified anti-Ex6P IgG was added for 1 h at 37 °C, and the antigen-antibody complexes were revealed with anti-rabbit IgG conjugated with peroxidase. The complexes were detected by orthophenylene diamine. The experiment was repeated twice with similar results.
iodinated V189 (50-, 38-, and 34-kDa isoforms) at 4 or 37 °C for 2 h. The specific binding was determined in the presence of an excess of unlabeled 38-kDa V189 because its binding to Flk-1 and Flt-1 has been demonstrated in the previous experiments. As shown in Fig. 6A, V165 bound ACE cells slightly more at 4 than at 37 °C (360 and 270 pg/10^5 cells, corresponding to 4,900 and 3,700 sites/cell, respectively). Only a low amount of 50-kDa V189 and Pl-V189 bound to ACE cells (240 and 640 sites/cell, respectively) as compared with uPA-V189, which could bind to 3,100 sites/ACE cell. The incubation of all of these V189 isoforms at 37 °C provided results similar to those obtained at 4 °C, suggesting that V189 maturation did not occur at the target cell level when added exogenously.

Stimulation of endothelial cell proliferation was evaluated using the same target cell. Half-maximal stimulation of ACE cell proliferation was obtained for a V165 concentration of 0.4 ng/ml. As shown in Fig. 7A, 38-kDa V189 and uPA-cleaved V189 were as potent as V165 in inducing a mitogenic effect on ACE cells (ED_{50} values of 0.9 and 1 ng/ml, respectively). In contrast, the plasmin fragment was less active (ED_{50} value of 24 ng/ml). Native V189 did not induce any proliferation. The same experiments were performed on pgsA-745 cells transfected with V189 expression vector. After confluency, the cells were incubated with heparin and leupeptin, and V189 was eluted as a 52-kDa mature protein as described in the legend to Fig. 2C. As shown in Fig. 7B, V189 did not promote cell proliferation unless it had been cleaved by uPA. As expected, uPA affected neither the basal proliferation nor that induced by V165 or FGF2. These experiments suggested that V189 was synthesized as an inactive pro-VEGF growth factor that required cleavage by serine proteases to elicit biological actions on endothelial cells.

**DISCUSSION**

The present data show that V189 is synthesized and accumulated in cell membranes as an inactive mitogen that can be cleaved to mature homodimers in the extracellular environment by the serine proteases urokinase and plasmin.

It seems, therefore, that the 24-amino acid insertion of V189 encoded by exon 6 contains information for both extracellular matrix or membrane retention and proteolytic maturation, which differs from that encoded by exons 5 and 7 of the VEGF gene. These observations agree with the data of Houck et al. (11), who demonstrated that a mutant of the longest form, V206, lacking the 24-amino acid insertion but containing the 17-amino acid insertion unique to V206, was efficiently released from the producing cells. Indeed, we hypothesize that native V206 would also be able to undergo a maturation process similar to that described here for V189. The relationships between intact V189 and the cell membranes are apparently complex and involve more than electrostatic interactions between anionic charges of the heparansulfates and the basic sequences encoded by the exon 6 and 7 as suggested by Houck et al. (1992). Our data suggest the presence of another type of binding site, which would be sequence-specific since V189 is equally released by Ex6P and the less cationic Ex6sP peptide containing the same amino acid 126–134 sequence but is not significantly released by the isocationic peptide SP containing a different sequence. The purification of a 60–72-kDa binding site for exon 6-encoded peptide has recently been reported (28), which may explain our results. Data from flow cytometry analysis show that the disruption of the interactions between V189 and any of the cell membrane binding sites achieved by the
addition of either heparin or Ex6P was sufficient to release V189. However, in both cases V189 appeared to be spontaneously cleaved in the extracellular environment of heparan sulfate-deficient cells, and the maturation process was uPA-dependent, since both the inhibition of its enzymatic activity and its immunoneutralization prevented the release of the 42/40-kDa V189. It appears, however, that enzymatic cleavage is not required for V189 release, since the addition of heparin to uPA-inactivated cells resulted in the release of only the native 52-kDa V189 isoform. Conversely, the inhibition of plasmin activity did not affect V189 release or maturation, confirming that uPA interaction with V189 was direct and did not result from activation of plasmin. It would seem from the resulting molecular weight and the fact that post-translational glycosylation has been characterized on Asp^100^Ala^111^ (29) that the proteolytically clipped 40-kDa V189 species most probably represents the amino-terminal part of the molecule. It has been recently demonstrated that the plasmin cleavage site is localized as Arg^110^Ala^111^ (29). This is consistent with the observation that plasmin cleavage did not lead to the presence of a 12-kDa fragment, since the sequence 111–165 does not contain any tyrosine residue. Although V189 contains a tyrosine residue encoded by exon 6 (Tyr^134^), no iodinated band was detected in the 16-kDa range, suggesting that either Tyr^134^ is not iodinated or that plasmin cleaves V189 in shorter fragments that would not be detected under our experimental conditions. Several lines of evidence demonstrate that plasmin and uPA generate distinct molecular species from V189. In contrast to Pl-V189, uPA-V189 bound to heparin-Sepharose and to IgG directed against a synthetic peptide 119–134. Since V165 is not cleaved by uPA, this cleavage might occur within the sequence encoded by exon 6, which is consistent with the difference of 4 kDa observed between the two homodimeric molecular species. Processing into 38- or 34-kDa homodimers is a prerequisite for V189 to become competent to bind to Flk-1 and to induce cell proliferation. Both isoforms could bind to each VEGF receptor constitutively expressed in heparan sulfate-deficient CHO cells, although Pl-V189 exhibited a 3–4-fold reduced affinity for both VEGF receptors. These results contrast with the observation that iodinated 50-kDa form and Pl-V189 bound only to a low number of binding sites on ACE cells, presumably Flt-1 receptors, which are expressed in ACE cells (30). Accordingly, Pl-V189 elicited a 50-fold reduced mitogenic effect on ACE cells as compared with uPA-V189. Similar observations have been made in a recent report (29) ascribing the reduction of the mitogenicity of plasmin-cleaved V165 to the lack of interaction of the basic sequence encoded by exon 7 with proteoheparan sulfates. The nature of these proteoheparan sulfates has not yet been characterized, but their removal impairs the binding of the 121 isoform of VEGF, which in addition is also a heparin-binding protein (31). This hypothesis might also explain that the presence of the basic sequence encoded by exon 6 would stabilize V189 through interactions with proteoheparan sulfates.

A wide number of biologically active peptides are synthesized as inactive precursors. Another growth factor, the hepatocyte growth factor/scatter factor is also generated by uPA cleavage of a precursor into a mature αβ heterodimer (15, 16). However, the mature αβ heterodimer does not contain a sequence similar to that encoded by exon 6 of V189, and this maturation can take place both at the producer and the target cell level. uPA is an extracellular serine endoprotease critically involved in tissue remodeling, cell migration, invasion, and metastasis as well as in the control of hemostasis by activation of plasminogen present in high concentrations in extracellular fluids to plasmin, a fibrinolytic enzyme with broad specificity. Our findings dem-

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