Cell Release of Bioactive Fibroblast Growth Factor 2 by Exon 6-encoded Sequence of Vascular Endothelial Growth Factor*

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Vascular endothelial growth factor (VEGF) is a potent angiogenic factor which is synthesized and secreted by many differentiated cells in response to various stimuli including hypoxia and growth factor exposure. Alternative splicing of vascular endothelial growth factor mRNA results in three distinct molecular forms: V189 and V165 or V121 which lack the exons 6 and 6, respectively. To clarify the functions of the 24-amino acid insertion, the biological activity of V189 was compared with that exerted by purified recombinant V189 and a synthetic peptide designed on the sequence encoded by exon 6 (Ex6P). V189 and Ex6P, but not V165, induced cell proliferation on corneal endothelial cells cultured in vitro. These effects were due to the release of fibroblast growth factor 2 (FGF2) stored in the extracellular matrix but not to direct interactions with FGF receptors since V189 was inefficient on heparan sulfate-deficient cells expressing constitutively FGF-R1. Moreover, corneas incubated ex vivo with Ex6P solubilized 10-fold more FGF2 than a isocationic peptide containing a scrambled sequence. Ex6P elicited an angiogenic response in a corneal pocket assay which was totally inhibited by addition of anti-FGF2 IgG. Moreover the angiogenic response to V189, but not to V165, was inhibited by FGF2 immunoneutralization. These findings demonstrate that the presence of the exon 6-encoded sequence confers VEGF with the ability to exert its biological effects through FGF2 signaling pathways.

Sprouting of new capillaries from preexisting vessels, or angiogenesis, occurs in several physiological or pathological conditions such as tumor progression, diabetic retinopathy, or rheumatoid arthritis (1). This local hypervascularization is thought to result from the release by the tissues of growth factors interacting with their receptors on endothelial cells which in turn migrate, proliferate, and differentiate into new capillaries. Several growth factors such as fibroblast growth factor 1 and 2 (FGF1, FGF2) or vascular endothelial growth factor (VEGF) are angiogenic in vivo. VEGF (2) also called vasculotropin (3) or vascular permeability factor (4, 5) stimulates in vitro the proliferation of vascular endothelial cells (2–4), lymphocytes (6, 7), and retinal pigment epithelial cells (8). Molecular cloning of human cDNA has shown that the factor is in fact composed of multiple species which are generated after alternative splicing of a single RNA transcribed from a single gene (9–12). All transcripts encode a 26-amino acid hydrophobic signal sequence and have identical mature amino termini. The transcripts encoding the 165- and 121-amino acid forms are freely soluble in the tissue culture medium. In contrast a longer transcript encodes a 189-amino acid form with a 24-amino acid peptide corresponding to exon 6 of the gene which appears to bind to heparan sulfate proteoglycans in the extracellular matrix and the cell membrane (13). These authors suggest that V189 sequestration would provide a reserve of biologically active growth factor from which the V189 species can be released by heparin or through proteolysis following plasminogen conversion into plasmin. VEGF binds to two different receptors Flt-1 (14) and KDR (15) or its murine homologue Flk-1 (16). The binding domains of VEGF to Flt-1 and KDR/Flk-1 have been identified as the sequences 63–67 and 82–86, respectively (17). We recently demonstrated that native V189 binds to Flt-1 but requires a cleavage by urokinase located within the exon 6-encoded sequence to bind to KDR/Flk-1 and to exert a mitogenic effect on vascular endothelial cells (18). However the mitogenic effect of the shorter form generated by plasmin cleavage between Arg110 and Ala111 was reduced 10–100-fold as compared with that of urokinase-matured V189 (18) or V165 (19). Because these results suggested that exon 6 acted as a mitogenic enhancer, we then compared the physiological roles exerted by V165, V189, and a synthetic peptide designed on the sequence encoded by exon 6 (Ex6P). It appeared that, in contrast to V165, V189 and Ex6P could exert a mitogenic activity, even in the absence of KDR/Flk-1 activation, providing the target cells expressed and stored FGF2 in their cell membranes and extracellular matrix. The use of neutralizing antibodies against FGF2 showed that this biological activity was due to the release of FGF2 bound to cell membranes in vitro, ex vivo, and in vivo. Accordingly V189, in contrast to FGF2, did not promote cell migration in CHO cells transfected with FGF-R1. These findings demonstrate that the presence of the exon 6-encoded sequence confers VEGF with the ability to exert its biological effects through FGF2 signaling pathways.

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1 The abbreviations used are: FGF, fibroblast growth factor; Ex6P, synthetic peptide RGKGRGKRRKRRKKRSRY; SP, synthetic peptide RKGRKRGKRRKRRKKRSRY; V189, vascular endothelial growth factor 165; V189, vascular endothelial growth factor 189; uPA-V189, urokinase-cleaved V189; PI-V189, plasmin-cleaved V189; PIGF, placenta growth factor 152; FBAE, bovine fetal aortic endothelial cells; BCE, bovine corneal endothelial cells; pgsA-745, heparan sulfate-deficient CHO cells; BHK, baby hamster kidney cells; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; EGF, epidermal growth factor.
EXPERIMENTAL PROCEDURES

Materials—Electrophoresis reagents were from Bio-Rad, chromatography reagents from Pharmacia, and Na\textsubscript{2}EDTA from Amersham. Cell culture reagents were from Life Technologies, Inc. Cell culture trays were from Costar. Recombinant human V165 and V189 were synthesized in Sf9 cells infected with a recombinant baculovirus containing the V165 or V189 cDNA and purified by cation exchange and heparin affinity chromatography (18). Two molecular species were purified from V189-infected Sf9 cells: the 50-kDa native V189 and the 38-kDa matured V189. The purity of V165 and V189 was ascertained >95% by SDS-polyacrylamide gel electrophoresis. Native V189 was further cleaved by urokinase or plasmin as already described and referred to as uPA-V189 and Pl-V189, respectively. Recombinant FGF2 was prepared in Escherichia coli as described (18). Anti-FGF2 IgG were prepared as already described (20). [\textsuperscript{3}H]Thymidine ([\textsuperscript{3}H]Td) was from Amersham. The peptides corresponding to the sequence 119–134 (Ex6P, RGKGKQKRRKKRRSRY) or a scrambled sequence (SP, RKGKRQK-RKGKSKYR) were synthesized by a solid-phase technique.

Growth Factor Bioassays—Bovine endothelial cells cultured from fetal aortas (FBAE) or cornea (BCE) were grown in Dulbecco’s modified Eagle’s medium supplemented with 2 mM l-glutamine, 50 units/ml penicillin, 50 \textmu g/ml streptomycin, 0.25 \textmu g/ml fungizone, and 10% newborn calf serum (FBAE) or 15% fetal calf serum (BCE). Stock cultures received 1 ng/ml FGF2 every other day (21). Baby hamster kidney (BHK) and xyleneesterase-deficient pg47-75 CHO cells expressing FGF-R1 (22) were cultured in Dulbecco’s modified Eagle’s medium-P12 medium supplemented with 10% bovine fetal calf serum, 50 units/ml penicillin, 50 \textmu g/ml streptomycin, 0.25 \textmu g/ml fungizone, and 2 mM l-glutamine. Cells were transfected with a mixture of 10 \mu g of pSV7d expression vectors carrying the Flt-1 or the Flk-1 coding sequences and 1 \mu g of pSV2 expression vector carrying the neo resistance gene with the Lipofectin reagent (Life Technologies, Inc.). Stable integrants were selected using 500 \mu g/ml G418; cloning was carried out by colony isolation using a Pasteur pipette. Transfectants were screened by their ability to specifically bind iodinated VEGF.

Migration wound assays were performed on growth-arrested cells seeded in 24-well cluster plates. The cell monolayers were incubated in serum-free medium with the modulators and 1 \muCi of [\textsuperscript{3}H]thyminidine (47 Ci/mmol) for 4 h. The insoluble material was precipitated for 10 min with 10% trichloroacetic acid, neutralized, and dissolved in 0.2 M NaOH, and the radioactivity was counted in a scintillation counter. Migration wound assays were performed on confluent cells as described by Sato and Rifkin (23).

FGF2 Binding Assays—4 \mu g of FGF2 were iodinated by the IODOGEN procedure according to Ref. 24. The specific activity averaged 100,000 cpm/\mu g. Cross-linking was performed as described. Cell cultures were washed twice in ice-cold binding medium (Dulbecco’s modified Eagle’s medium, 20 mM HEPES, pH 7.5, 1 mg/ml gelatin) and incubated (with the medium with 10 ng/ml [\textsuperscript{125}I]FGF2, 1 \mu g/ml unlabeled FGF2, V165, or V189 or 50 \mu g/ml Ex6P or SP) for 30 min at room temperature and stopped with 0.2 M glycine, pH 7.4. The cell pellet was resuspended in 1 \times sample buffer, reduced (0.05 M \beta-mercaptoethanol), and boiled. Cells in parallel cultures were counted, and samples corresponding to 10\textsuperscript{6} cells per lane were loaded on a SDS-7% polyacrylamide gel. Gels were stained with Coomassie Blue, dried, and autoradiographed.

In parallel experiments 2 ng/ml iodinated FGF2 was allowed to bind to cell monolayers, washed, and then incubated for 3 h more with various dilutions of unlabeled peptides. The FGF2 eluted from cell membranes was quantitated in a y counter.

Ex Vivo Elution of FGF2 from Bovine Corneal Strontia—20 corneas were dissected from bovine eyes collected at a local slaughterhouse. The tissue was minced and sonicated with 100 ml of 0.05 M Tris, 0.15 M NaCl, pH 7.2. Each half of the homogenate received 50 \mu g/ml Ex6P or SP and was incubated overnight at 4 °C under constant stirring. After centrifugation at 20,000 \times g for 20 min, the soluble extracts were dialyzed (cut-off 8,000 Da) against the same buffer to remove the peptides and loaded on 1-ml heparin-Sepharose columns, and the retained material was eluted by a 15-ml gradient of 0.5–2 M NaCl diluted in the same buffer. FGF2 was measured in the fractions by radioimmunoassay as already described (20).

Corneal Pocket Angiogenesis—Slow releasing implants of hydrogel (2 \times 1 mm), were rehydrated with 2 \mu l of PBS containing 50 \mu g of bovine serum albumin and 200 ng of growth factors or 1 \mu g of synthetic peptide in the presence or absence of 30 \mu g of anti-VEGF or anti-FGF2 IgG. The specificity of anti-FGF2 IgG was ascertained by radioimmunoassay which showed that neither synthetic peptides nor V189 or V165 could cross-react with this antibody. These implants were inserted in the corneal stroma 2 mm away from the limbus (25). After 14 days the neovascularization was assessed by direct examination with a slit lamp and scored according to a 5-grade scale (0, no vessel; 1, vessel length <1 mm; 2, vessel length <2 mm; 3, vessel length <3 mm; 4, vessels surrounding the pellet). The data are presented as mean score ± S.E. for each condition. Statistical analysis was performed according to the Fisher test.

RESULTS

uPA-V189 Is Mitogenic for KDR/Flk-1-deficient Cells—We have previously demonstrated (18) that V189 binds to the VEGF receptor Flt-1 and requires a cleavage with urokinase (uPA-V189) or plasmin (Pl-V189) to bind and activate the alternate receptor KDR/Flk1. We compared their bioactivities to that of V165 on several cell lines. Surprisingly, uPA-V189 induced a mitogenic effect on bovine corneal endothelial (BCE) cells (Fig. 1) although these cells do not express KDR/Flk-1 (26), the VEGF receptor mediating the VEGF-dependent mitogenic effect (27, 28). Pl-V189 was not mitogenic. EC\textsubscript{50} values corresponding to FGF2 and uPA-V189 were 0.6 and 0.8 ng/ml, respectively.

Anti-FGF2 IgG abolished thymidine incorporation induced by FGF2 and uPA-V189 on BCE cells and significantly reduced that induced on FBAE cells (Fig. 2). In contrast the mitogenic effect of V165 on BCE cells was not affected by anti-FGF2 IgG but was abolished by anti-VEGF IgG.

Effect of uPA-V189 and Ex6P on FGF2 Cross-linking to Cell Membranes—in another set of experiments it appeared that FGF2, V165, and uPA-V189 induced a chemotactic effect on BCE cells (Fig. 3A). Surprisingly Ex6P exerted a chemotactic effect as a function of the dose added. Half-maximal and maximal stimulation were observed for 0.7 and 3.5 \mu g/ml, respectively (Fig. 3B). The dose-response curve was biphasic and decreased at concentrations above 20 \mu g/ml. Conversely SP did not induce a significant chemotactic effect. Immunoneutralization of FGF2 also abolished the chemotactic effect of Ex6P and FGF2 and significantly inhibited that exerted by uPA-V189 (Fig. 3A).
Although native V189 was not mitogenic for BCE cells, it induced a chemotactic activity. We examined whether this effect was mediated through direct interactions with FGF2 receptors or with VEGF receptors. V189 contains two cationic domains encoded by exons 6 and 7 which are both required for its membrane anchor. These basic domains are involved in V189 binding to a still unknown membrane binding site and to proteoheparan sulfate proteoglycans, respectively. For a better understanding of the role of the sequence encoded by exon 6, we transfected heparan sulfate-deficient pgsA-745 CHO cells with FGF-R1, Flt-1, or Flk-1. Iodinated FGF2 was cross-linked to cells expressing constitutively FGF-R1 on a molecular species of 160 kDa. Subtraction of the molecular mass of FGF2 from the size of the cross-linked complex yields gave an estimated molecular mass of 140 kDa. Excess unlabeled FGF2, Ex6P, SP, and V189, but not V165, blocked the formation of these cross-linked complexes (Fig. 4).

Nontransfected cells did not migrate in response to any growth factor. Flt-1-, but not Flk-1-, expressing cells migrated in response to V165, PlGF (placenta growth factor, 152-amino acid splice variant), or V189, thus demonstrating that Flt-1 is the VEGF receptor mediating cell migration. V189, in contrast to FGF2, could not induce the migration of FGF-R1-expressing pgsA-745 cells (Fig. 5). In contrast none of the Ex6P or SP peptides could induce migration on pgsA-745 cells expressing constitutively Flt-1 or FGF-R1 (data not shown).

In Vitro Release of FGF2 from Cell Membranes—To confirm that V189 inhibited the cross-linking of iodinated FGF2 to its receptors through competition with FGF2 rather than through direct interactions with FGFRα, we determined whether it could release FGF2 from its cellular binding sites. Iodinated FGF2 was allowed to bind to BCE cells, and then the cell monolayers were rinsed and incubated in the cold with various peptides or growth factors. FGF2 and uPA-V189 released up to 60% of iodinated FGF2 as a function of the dose (IC50 corre-
sponding to 0.7 and 12 nM, 12 and 600 ng/ml, respectively. In contrast similar concentrations of V165 or Pl-GF did not release any iodinated FGF2 from cell membranes (Fig. 6A).

Similar concentrations of uPA-V189 released iodinated FGF2 when BHK, wild-type, or FGFR1-transfected pgsA-745 CHO cells were used as target cells (Table I). However uPA-V189 was not mitogenic for these cells, which do not express FGF2, suggesting that the proliferation observed in FBAE and BCE cells resulted from FGF2 release from their membranes and extracellular matrix. Ex6P and SP released iodinated FGF2 as a function of the dose, and half-maximal release was obtained for concentrations of 0.1 and 1.5 nM, respectively (Fig. 6B). Although Ex6P could release almost 80% of the bound FGF2, SP did not remove more than 45%, suggesting the existence of several FGF2 binding sites. This sequence-related effect on FGF2 release was confirmed by the observation that the shorter peptide containing the sequence 126–134 amino acids encoded by exon 6 released as much FGF2 as Ex6P with a IC50 of 10 nM (not shown).

Ex Vivo Release of FGF2 by Ex6P—Fresh bovine corneas were homogenized in Tris buffer and incubated with Ex6P or SP, and the material extracted was chromatographed on heparin-Sepharose affinity columns. The fractions were assayed for their FGF2 content by radioimmunoassay. As shown in Fig. 7, FGF2 immunoactivity was eluted at 1.6 M NaCl by Ex6P (120 ng/cornea). In contrast SP released 10-fold less immunoactive FGF2 than Ex6P.

In Vivo Release of FGF2 by Ex6P—To confirm that the FGF2 immunoactivity released from corneas by the synthetic peptides was bioactive, their angiogenic activity was assayed in a corneal pocket assay. As shown in Table II the implants containing the vehicle alone did not exhibit any angiogenic response, whereas Ex6P appeared to be a strong inducer of angiogenesis (score = 3.14 ± 0.26 for 10 μg), and SP had a weak effect (score = 1.42 ± 0.20 for 10 μg). 200 ng of V165, uPA-V189, or FGF2 induced similar angiogenic activities (scores of
The soluble extracts were dialyzed and loaded on heparin-Sepharose columns, and the retained material was eluted by a 15-ml gradient of 0.5–2 M NaCl diluted in the same buffer. FGF2 was measured in the fractions by radioimmunoassay.

**TABLE II**

Neutralization of the angiogenic activities of Ex6P and SP by anti-FGF2 IgG

Slow releasing implants (2 × 2 mm) were rehydrated with 10 µg of Ex6P or SP in the absence or presence of 30 µg of anti-FGF2 IgG or anti-V165 IgG diluted in 2 µl of PBS containing 50 µg of bovine serum albumin. These implants were inserted in the corneal stroma 2 mm away from the limbus. Neovascularization was assessed after 14 days by direct examination using a slit lamp, and the eyes were photographed. Results are expressed as the mean ± S.E. of the neovascularized surface.

| Modulators | n  | Score > 1 | Mean score | S.E. |
|------------|----|-----------|------------|------|
| Vehicle    | 19 | < 5       | 0.05       | 0.05 |
| Ex6P       | 22 | 100       | 3.14       | 0.26 |
| Ex6P + anti-FGF2 | 7 | 28       | 0.39       | 0.31 |
| Ex6P       | 7 | 28       | 0.57       | 0.29 |
| SP         | 5 | 100       | 1.42       | 0.20 |
| FGF2, 0.2 µg | 22 | 100       | 2.47       | 0.07 |
| FGF2 + anti-FGF2 | 10 | 25       | 0.52       | 0.21 |
| V165, 0.2 µg | 37 | 100       | 3.28       | 0.17 |
| uPA-V189, 0.2 µg | 10 | 100       | 3.12       | 0.12 |

3.28 ± 0.17, 3.12 ± 0.12, and 2.47 ± 0.07, respectively). The angiogenic activities of FGF2, Ex6P, and SP were totally blocked by anti-FGF2 IgG (scores of 0.52 ± 0.21, 0.39 ± 0.31, and 0.57 ± 0.29, respectively) and remained unaffected by anti-V165 IgG (not shown). Surprisingly, although V189 was not mitogenic in vitro for vascular endothelial cells, it exerted a strong angiogenic effect in the corneal pocket assay (Fig. 8). Neutralizing anti-VEGF IgG, but not anti-FGF2 IgG, could totally block the angiogenic activity of V165. The angiogenic activity of V189 was in contrast inhibited but not totally abolished by each of the neutralizing IgGs.

**DISCUSSION**

The data presented in this paper show that, besides information for extracellular matrix or membrane retention, the 24-amino acid insertion of V189 also contains information to activate the FGF2 signaling pathways in vitro as well as in vivo.

VEGF is the angiogenic growth factor which seems the most vessel-specific in vivo. Nevertheless VEGF exerts mitogenic effects on several cultured cells such as vascular endothelial cells, T lymphocytes, retinal pigment epithelial cells (2–8), and stromal cells cultured from neonatal hemangiomata (28). Alternative splicing of the pre-mRNA generates 3 distinct isoforms. We had previously demonstrated that V189 is secreted by heparan sulfate-deficient CHO cells and further sequestered on two different binding sites. Its release requires a cleavage by urokinase which allows V189 to bind to KDR/Flk-1 and to exert a mitogenic effect on vascular endothelial cells (18). However the shorter form generated by plasmin cleavage between Arg110 and Ala111 exerted a mitogenic effect that was reduced by 50-fold compared with that of urokinase-matured V189. Since both PI-V189 and uPA-V189 bound equally to the VEGF receptors, these results suggest that exon 6 behaved as a mitogenic enhancer. Surprisingly uPA-V189 could induce thymidine incorporation on corneal endothelial cells despite the fact that these cells did not express KDR/Flk-1 (26), the VEGF receptor mediating cell proliferation (27, 28). This observation suggested that uPA-V189 mitogenic activity was exerted through the activation of other growth factor pathways. Several recent studies have pointed out that V165 and FGF2 signaling pathways might synergize to trigger angiogenesis in vitro and in vivo (29). However we did not notice any synergy between V165 and FGF2 for the proliferation of cells cultured on plastic, confirming our previous results (30), but we observed that uPA-V189-dependent proliferation of BCE cells was blocked by neutralizing anti-FGF2 IgG. We then focused on the possible interactions of VEGF and FGF2.

FGF2 interacts with specific high affinity receptors and heparan sulfate proteoglycans to form trimolecular complexes on the cell surface (31). The FGF receptors, like the VEGF receptors, are tyrosine kinases which contain three immunoglobulin-like domains in the extracellular portion of the molecule, instead of seven for the VEGF receptors. The binding of FGF2 to heparan sulfate moieties of proteoglycans increases its affinity for the FGF receptor tyrosine kinases (32) and allows its storage in the cell membranes and extracellular matrix (33). The inhibition of iodinated FGF2 cross-linking to its receptors by peptides containing the basic sequence encoded by exon 6 of VEGF might result from a direct activation of the FGF receptors or from competition with FGF2. The first possibility was ruled out by the finding that V189, uPA-V189, or Ex6P could not induce cell migration or proliferation on BHK cells which do not synthesize FGF2. Moreover the use of heparan sulfate-deficient CHO cells expressing constitutively the VEGF receptors demonstrated that homodimerization of Flt-1 obtained by PIGF or V165 was sufficient to trigger cell migration. In contrast to the report of Waltenberger et al. (27), we found that
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Flk-1 activation was not sufficient to promote chemotaxis. Interestingly native V189 and the PiGF 2 splice variant, which both contain a basic sequence encoded by exon 6, could bind to Flk-1 and induce cell migration on these cells. The finding that V189 was not chemotactic for FGF-R1-expressing pgA-A745 CHO cells also favored the second hypothesis. In fact uPA-V189 and Ex6P, but not V165, could (i) compete with FGF2 binding to its receptors, (ii) release FGF2 in vitro and ex vivo, (iii) induce a mitogenic effect only on target cells which express FGF2 and store it in their membranes and extracellular matrix, and (iv) have anti-FGF2 neutralizing antibodies block their mitogenic and angiogenic activities. In addition the discrepancy observed between the lack of mitogenic activity exerted in vitro by native V189 and its strong angiogenic activity in vivo suggests that its maturation occurs in vivo.

Several reports (34, 35) have characterized the interactions of a synthetic peptide, representing the alternatively spliced exon 6 of PDGF A-chain, with a large population of cell surface binding sites. The amino acid sequences of exon 6 of the PDGF A-chain and exon 6 of the VEGF are closely related (36) with 6 consecutive residues in common but in the reverse sense (KKRRKK instead of KRRKRR). Both groups concluded in the consecutive residues in common but in the reverse sense (KKRRKK instead of KRRKRR). Both groups concluded in the

FIG. 9. Schematic representation of the role of V189 processing on FGF2 release.

These results demonstrate the dual mechanism of the action of V189 schematized on Fig. 9. The folding of native V189 would mask its binding domain to Flk-1 (amino acids 82–86, Ref. 17), but its binding domain to Flt-1 (amino acids 63–67, Ref. 17) is functional. The sequences encoded by exons 6 and 7 mediate V189 binding to CRS-BP1 (Ref. 38) and to proteoglycan sulfate (Refs. 19 and 40), respectively. The binding to these two distinct sites anchors V189 in the plasma membrane (Ref. 18). When V189 is cleaved by urokinase it can interact with the two VEGF receptors and release FGF2 from its storage sites, which in turn can activate the FGF receptors. When V189 is cleaved by plasmin it does not interact with CRS-BP1 or proteoglycan sulfates and does not release FGF2. It might be of interest to determine if other basic sequences encoded by exons 6 of the A or B chains of PDGF or PiGF can also release bioactive FGF2 from extracellular matrix.

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15. Terman, B. I., Dougher-Vermazen, M., Carrion, M. E., Dimitrov, D., Armellino, molar range, a value close to the $K_d$ of FGF2 for heparan sulfate proteoglycans (37). Ex6P and SP released iodinated FGF2 bound to BCE or FBAE cells as a function of the dose, but the IC$_{50}$ corresponding to Ex6P was 15-fold lower and the total amount released was almost twice that released by SP. These data suggest that beside competition of cationic peptides with heparan sulfates, other membrane binding sites would interact with the exon 6-encoded sequence, possibly through a protein-protein interaction. Such a binding site for the VEGF exon 6-encoded sequence has been recently purified as 69–75-kDa “CRS-BP1” proteins (38). In fact we observed that the exon 6-encoded sequence conferred VEGF with the ability to bind to heparan sulfate-deficient cells (18). Another group recently identified a splice variant of VEGF lacking the exon 7 (39). They also observed that this VEGF variant was able to bind to the extracellular matrix even after pretreatment with chlorate which prevents the sulfation of heparan proteoglycans. The finding that Ex6P released FGF2 from fresh corneas ex vivo was physiologically relevant since the peptide alone was sufficient to elicit corneal angiogenesis through this indirect mechanism involving FGF2 signaling pathways.

The results demonstrate the dual mechanism of the action of V189 schematized on Fig. 9. The folding of native V189 would mask its binding domain to Flk-1 (amino acids 82–86, Ref. 17), but its binding domain to Flt-1 (amino acids 63–67, Ref. 17) is functional. The sequences encoded by exons 6 and 7 mediate V189 binding to CRS-BP1 (Ref. 38) and to proteoglycan sulfate (Refs. 19 and 40), respectively. The binding to these two distinct sites anchors V189 in the plasma membrane (Ref. 18). When V189 is cleaved by urokinase it can interact with the two VEGF receptors and release FGF2 from its storage sites, which in turn can activate the FGF receptors. When V189 is cleaved by plasmin it does not interact with CRS-BP1 or proteoglycan sulfates and does not release FGF2. It might be of interest to determine if other basic sequences encoded by exons 6 of the A or B chains of PDGF or PiGF can also release bioactive FGF2 from extracellular matrix.
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