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Characterization of the Extracellular Domain in Vascular Endothelial Growth Factor Receptor-1 (Flt-1 Tyrosine Kinase)

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Flt-1 tyrosine kinase, vascular endothelial growth factor (VEGF) receptor-1, binds VEGF and a new VEGF-related ligand, placenta growth factor, but KDR/Flk-1 (VEGF receptor-2) binds only VEGF. To characterize the functional regions in the Flt-1 extracellular domain such as the ligand binding region and the dimer formation of the receptor, we constructed a series of mutants of the Flt-1 extracellular domain as soluble forms in a baculovirus system. We found that a region carrying the N-terminal 1st to 3rd immunoglobulin (Ig)-like domains of Flt-1 binds both ligands with high affinity. However, for dimer formation of soluble Flt-1, a region further downstream in the Flt-1 extracellular domain was required. Mutant Flt-1 receptors expressed in COS cells confirmed the requirement of the 4th to 7th Ig region for the activation of Flt-1 tyrosine kinase. Soluble Flt-1 carrying the N-terminal 1st to 3rd Ig region suppressed VEGF-dependent endothelial proliferation *in vitro* to the same level as the larger forms of soluble Flt-1, suggesting that the binding of one soluble Flt-1 molecule to one subunit of the VEGF homodimer may be sufficient to block the VEGF activity.

Key words: VEGF — Angiogenesis — Tyrosine kinase — Flt-1

Proliferation, differentiation and tubular formation of endothelial cells are fundamental processes of angiogenesis.[1,2] The endothelial cell growth factor VEGF (vascular endothelial growth factor), also designated as VPF (vascular permeability factor), has roles in a variety of processes involving angiogenesis under normal conditions, such as embryogenesis, corpus luteum formation and placental growth, as well as under pathological conditions, such as diabetic retinopathy, rheumatoid arthritis and tumor angiogenesis.[3-5]

We isolated the tyrosine kinase receptor *flt-1* (*fms*-like tyrosine kinase) gene that encodes the 7-Ig domain containing the extracellular region and a tyrosine kinase carrying an insert of about 70 amino acids (kinase insert) in the middle of this domain.[6] Flt-1 and its related tyrosine kinase KDR/Flk-1 are high-affinity receptors for VEGF.[7-10] Furthermore, a VEGF-related ligand PlGF (placenta growth factor) highly expressed in placental tissue binds Flt-1 but not KDR/Flk-1.[11-14] We also reported that the activation of tyrosine kinase in Flt-1 proceeds upon stimulation with both VEGF and PlGF, indicating that these ligands are functional for Flt-1.[14] However, 10- to 20-fold less activation of Flt-1 tyrosine kinase than KDR/Flk-1 was noted. Thus, Flt-1 is unique among tyrosine kinase receptors in terms of its very low level of tyrosine kinase activity.[15] The K_d value of the association of Flt-1 with VEGF was 2–10 pM, whereas that of PlGF was about 200 pM.[12-14] Very recently, Flt-1 has been reported to transduce signals for cell migration and tissue factor production of human monocytes.[16,17]

Mutational analysis of the VEGF indicates that the binding regions of VEGF for the two receptors are separately located on the molecule.[18] The results suggest that the critical amino acids in VEGF for the interactions with these two receptors are independent. Recently an initial characterization of the ligand-binding region in VEGF receptor has been reported using the chimeric protein method.[19]

In this study, we constructed deletion mutants of the extracellular domain of the Flt-1 without fusion to other peptides and examined the ligand-binding region and the sequence essential for dimer formation of the receptor. We found that the N-terminal 340 amino-acid stretch contains the critical sequences for high-affinity binding to the ligands, whereas the region further downstream is required for dimer formation.

MATERIALS AND METHODS

Reagents, cells, and viruses *Spodoptera frugiperda* (Sf9) cells, *Trichoplusia ni* (High Five) insect cells and baculovirus transfer vectors (pVL-1393) were purchased from Invitrogen (San Diego, CA). A linearized DNA of a mutant *Autographa californica* nuclear polyhedrosis viral DNA (BACULO GOLD) was from Pharmingen (San Diego, CA). TC-100 medium and tryptose phosphate broth were from Gibco BRL (Gaithersburg, MD). Serum-free medium (Ex-CELL 400) was from JRH Biosciences (Lenexa, KS). $Na^{125}I$ was obtained from ICN Biomedicals (Costa Mesa, CA), and ^{125}I-VEGF$_{165}$ was the product of Amersham (Buckinghamshire, England). Recombinant human basic FGF (fibroblast growth factor) was purchased from Oncogene Science (Uniondale,

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NY) and recombinant human PDGF (platelet-derived growth factor) was from Boehringer Mannheim (Mannheim, Germany). A portion of the purified human $VEGF_{165}$ used in this study was supplied by Dr. G. Neufeld (Technion-Israel, Inst. Tech., Haifa, Israel). Other $VEGF_{165}$ was obtained from the baculovirus system and purified on a heparin column. The rabbit polyclonal antibody #2 against the amino-terminal region of the human Flt-1 was provided by Dr. K. Ohsumi (Mitsubishi-Yuka BCL, Tokyo). The enhanced chemiluminescence detection kit was purchased from Amersham. N-Glycosidase F and O-glycosidase were from Boehringer Mannheim. Heparin-Sepharose and Sephadex G-25 columns were from Pharmacia (Uppsala, Sweden).

Construction of the deletion mutants of the Flt-1 extracellular domain To construct transfer vectors that direct the synthesis of portions of the extracellular domain of Flt-1, we used human *flt-1* cDNA cloned into the pUC 118 plasmid (*flt* #3–7).[6]

2N sFlt-1: An *Eco*R I-*Taq* I 918 bp fragment was excised from the *flt* #3–7, and subcloned into the *Eco*R I-*Not* I site of the multi-cloning region of pVL 1393 using a *Taq* I-*Not* I adaptor oligonucleotide containing an in-frame stop codon (5′-CGA CAA ACC AAT ATA ATC TAA GC-3′, 5′-GGC CGC TTA GAT TAT ATT GGT TTG T-3′).

3N sFlt-1: An *Eco*R I-*Taq* I 1263 bp fragment was cut out from the *flt* #3–7, and subcloned into the *Eco*R I-*Not* I site of the pVL 1393 using the same *Taq* I-*Not* I adaptor.

4N sFlt-1: An *Eco*R I-*Nde* I 1617 bp fragment was obtained from *flt* #3–7, and subcloned into the *Eco*R I-*Xho* I site of the shuttle vector pME18s-Neo[20] using a *Nde* I-*Xho* I adaptor containing an in-frame stop codon (5′-TAT TAA TGA TCT AGA ATG AC-3′, 5′-TCG AGT CAT TCT AGA TCA TTA A-3′). The resulting plasmid was cut with *Eco*R I and *Not* I, and the purified 1681 bp DNA fragment containing the 43 bp polylinker sequence from pME18s-Neo was subcloned into the *Eco*R I-*Not* I site of the pVL 1393.

5N sFlt-1: An *Eco*R I-*Hin*d III 1893 bp fragment from *flt* #3–7 was subcloned into the *Eco*R I-*Xho* I site of pME18s-Neo vector using a *Hin*d III-*Xho* I adaptor containing an in-frame stop codon (5′-AGC TTT TAA TGA TCT AGA ATG AC-3′, 5′-TCG AGT CAT TCT AGA TCA TTA AA-3′). The resulting plasmid was cut with *Eco*R I and *Not* I, and the purified 1959 bp fragment was subcloned into the *Eco*R I-*Not* I site of pVL 1393.

6N sFlt-1 is identical to 'sFlt-1' which is the soluble form of Flt-1.[21] To construct the 6N sFlt-1 transfer vector, we isolated the human Flt-1 cDNA fragment encoding the sFlt-1 carboxyl-terminal region from a human placenta cDNA library. This cDNA fragment carrying most of the coding region of sFlt-1 was subcloned into the *Eco*R I site of the pUC18 vector using an *Eco*R I linker. The clone (*flt* 7-3-1c) obtained was cut with *Eco*R I and ligated to an *Eco*R I-*Not* I-*Bam*H I adaptor (Takara Shuzo, Kyoto), then digested again with *Hin*d III and *Not* I. This *Hin*d III-*Not* I 0.5 kb sFlt-1 cDNA fragment bearing the carboxyl-terminal region was inserted into the *Eco*R I-*Not* I site of the pVL 1393 with the *Eco*R I-*Hin*d III 5′-1893 bp fragment from *flt* #3–7.

7N sFlt-1: We isolated its carboxyl-terminal region by means of the polymerase chain reaction (PCR) with the upstream primer that matches residues #1840–#1860 in human *flt-1* (5′-GGA ATC TAC ATT TGC ATA GCT-3′), and the downstream primer #2482–#2514 containing a *Not* I recognition sequence and an in-frame stop codon (5′-TTA TGC GGC CGC TTA TCC TTG AAC AGT GAG GTA-3′). The 674 bp PCR-amplified product was digested with *Hin*d III and *Not* I, then the purified 610 bp DNA fragment was subcloned into the *Eco*R I-*Not* I site of the pVL 1393 with the *Eco*R I-*Hin*d III 5′-1893 bp fragment from the *flt* #3–7.

Large-scale preparation and purification of the soluble Flt-1s These transfer vector DNAs were used for cotransfection into Sf9 cells along with the linearized baculovirus DNA "Baculo Gold" by liposome transfection. The recombinant viruses were amplified at 3-day intervals. High Five cells grown in serum-free medium Ex-CELL 400 were used to produce soluble Flt-1s. Cells were infected with high-titer virus stocks at a multiplicity of infection over 3. One hour after infection, cell cultures were changed to fresh Ex-CELL medium. Three days after infection, the culture media were harvested and centrifuged to remove debris. The supernatants were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on a 10% gel, followed by western blotting.

After filtration, supernatants containing sFlt-1 (20 ml) were loaded onto 1 ml volume of HiTrap-Heparin in a column equilibrated with 10 m*M* sodium phosphate buffer, pH 6.2. The column was washed with the same buffer, then with 10 m*M* sodium phosphate buffer containing 400 m*M* NaCl. For stepwise purification of 2N sFlt-1 the column was washed with 200 m*M* NaCl-phosphate buffer, and 2N sFlt-1 was eluted with 1 *M* NaCl in phosphate buffer. NaCl was removed from the sample using a Sephadex G-25 column. The partially purified proteins were identified by immunoblotting with rabbit α-Flt-1 #2 antiserum. The proteins were resolved by SDS-PAGE and silver-stained, then the protein amounts were measured by densitometry using bovine serum albumin (BSA) as a standard. The purity of these molecules was 30 to 80% (Fig. 2B).

Analysis of carbohydrates on the sFlt-1 proteins Purified sFlt-1 proteins (10–50 ng) were denatured by boiling in 0.4% SDS for 3 min and diluted 10-fold with reaction

buffer [10 m*M* sodium phosphate, pH 7.2/10 m*M* EDTA/0.05% NP-40]. The samples were digested with 0.2 U of N-glycosidase F and 0.5 mU of O-glycosidase or double-digested with 0.2 U of N-glycosidase F and 0.5 mU of O-glycosidase for 24 h at 37°C. The samples were then resolved by SDS-PAGE on 8% and 10% gel and western-blotted.

Iodination of PlGF-1 PlGF-1 was iodinated essentially as described.[15, 22] The specific activity of the product ^{125}I-PlGF-1 was 20,000–30,000 cpm/ng.

Ligand-binding assay VEGF was bound to sFlt-1s essentially as described.[20, 23] Purified sFlt-1 proteins (1 μg) were diluted to 10 ml with 10 m*M* sodium phosphate buffer, pH 6.2. Aliquots (100 μl) were attached to the surface of 96-well plates (Immulon 2, Dynatech) overnight at 4°C. The plates were then washed twice with phosphate-buffered saline, non-specific sites were blocked with binding buffer [Dulbecco's modified Eagle's medium (DMEM)/25 m*M* HEPES, pH 7.6/0.3% BSA] for 2–3 h at 20°C, and the plates were washed twice with the same buffer. Increasing amounts of ^{125}I-labeled VEGF were added to each well and the plates were incubated for 3 h at 20°C. The wells were washed three times with 200 μl of binding buffer, then the bound ^{125}I-labeled proteins were quantified in a gamma counter. All experiments were performed in triplicate. The values were analyzed according to Scatchard's procedure.[24] Competition was examined by incubating the sFlt-1-coated plates with a fixed concentration (1 ng/ml) of ^{125}I-labeled VEGF and non-radiolabeled ligand or sFlt-1 proteins.

Covalent cross-linking of ligand-sFlt-1 complexes Human ^{125}I-VEGF (0.48 ng) was incubated with 0.1, 1 or 10 ng of each purified sFlt-1 for 2 h at 4°C in 100 μl of binding buffer [20 m*M* HEPES, pH 7.6/150 m*M* NaCl/0.03% BSA]. Complexes were covalently cross-linked with 1 m*M* *bis*(sulfosuccinimidyl)suberate (Pierce Chemical, Rockford, IL) for 2 h at 4°C and the reactions were terminated by adding 1 μl of 1 *M* Tris-HCl (pH 7.6). The products were separated by SDS/8% PAGE, and the labeled bands were exposed to X-ray film.

Assay for inhibition of endothelial cell growth Rat liver non-parenchymal cells containing about 90% sinusoidal endothelial cells were prepared from rat liver by collagenase perfusion followed by differential centrifugation.[25, 26] The non-parenchymal cell (NP-cell) fraction was plated in Dulbecco's modified essential medium with 5% fetal bovine serum on collagen-coated 24-well plates at a density of 1×10^5 NP cells/well. Three hours after plating, the culture medium was replaced with the endothelial culture medium EGM-UV (Kurabo, Osaka) with various amounts of sFlt-1 proteins (0–250 ng/ml), followed by the addition of purified human $VEGF_{165}$ at 5 ng/ml. EGM-UV contains 2% fetal bovine serum, 10 ng/ml EGF, 1 mg/ml hydrocortisone and bovine brain extract, but cannot support cell proliferation or maintenance of sinusoidal endothelial cells without VEGF, as indicated in Fig. 8.[27]

Autophosphorylation of Flt-1 mutants expressed on COS cells The del 4N or the del 4–7N Flt-1 receptor cDNA was prepared by deletion of nucleotides #1231 to #1506 or #1231 to #2478, corresponding to amino acid residues #328 to #419 or #328 to #743, without additional nucleotides in the deletion sites. The wild-type (WT), del 4N or del 4–7N Flt-1 cDNA was inserted into the BCMGSneo

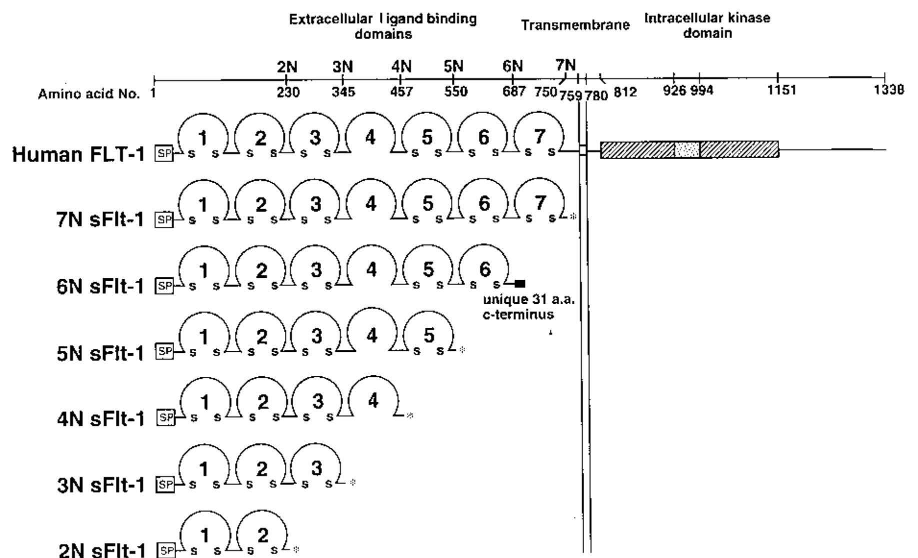

Fig. 1. Structural representation of the Flt-1 protein and recombinant soluble forms of its extracellular domain. The various proteins are schematically presented, and Ig-like domains are shown as loops. The predicted proteins are drawn to scale, and the functional domains are indicated at the top. Numbers 2 to 7 indicate the respective Ig-like domains, and the intra-domain disulfide bonds are shown as {-s s-}. SP, signal peptide; *, additional termination codon.

vector[15] and transfected into COS cells. These cells were selected with G418 and the clones overexpressing the desired proteins were screened by western blotting and established as clones #1–7 for WT, #19 for the del 4N and #8 for the del 4–7N Flt-1 COS cells.

These COS cell clones were grown in DMEM culture medium containing 10% calf serum to semi-confluence, then the cells were starved by changing the medium to DMEM-0.1% fetal calf serum. After 24 h starvation, the cells were stimulated with 50 ng/ml of VEGF for 5 min, and cell lysates were prepared using a lysis buffer.[15] The cell lysates were immunoprecipitated with anti human Flt-1 rabbit antiserum P1-2, then immunoblotted with the anti phosphotyrosine antibody PY20 plus 4G10 or with the anti human Flt-1 rabbit antiserum N2.

RESULTS

Preparation of soluble forms of Flt-1 To examine the binding sites and characteristics of the extracellular domain of the Flt-1, we constructed a series of deletion mutants of the Flt-1 amino terminal region and expressed them in the baculovirus-insect cell system for large-scale preparation (Fig. 1). As shown in Fig. 2, all constructs were efficiently produced and secreted into the culture medium as expected from the deletion of the trans-membrane and tyrosine kinase domains.

Since 6N Flt-1 which is identical to sFlt-1, a soluble Flt-1 encoded by an alternatively spliced *flt-1* mRNA, binds to heparin,[21] all the products from 2N to 7N forms were purified by heparin column chromatography. As indicated in Fig. 2A, all these products were purified in this manner, but the elution profiles differed. The 2N form was eluted at 400 m*M* NaCl, and the 7N form at 800 m*M* NaCl, and other forms were eluted between these concentrations.

The high binding affinity of these soluble Flt-1s (sFlt-1), including the 2N form, to heparin is of interest since KDR has essentially very weak or no affinity, even though it is highly glycosylated. We confirmed the glycosylation of all sFlt-1 forms by digesting them with glycosidases. As shown in Fig. 3, 2N sFlt-1 as well as the larger forms migrated to the positions of smaller molecules after digestion with N-glycosidase F, but not with O-glycosidase. Therefore, the results suggest that sFlt-1 is mostly N-glycosylated.

Binding analysis of the soluble Flt-1s with VEGF and PlGF Since the Flt-1 receptor expressed on fibroblast NIH3T3 cells or tumor cells binds both VEGF and PlGF with high affinity, we examined the region of the receptor responsible for VEGF binding and whether or not the binding regions for both ligands overlap.

As indicated in Fig. 4, except for 2N sFlt-1, all forms of sFlt-1 fixed on plastic plates bound ^{125}I-VEGF with

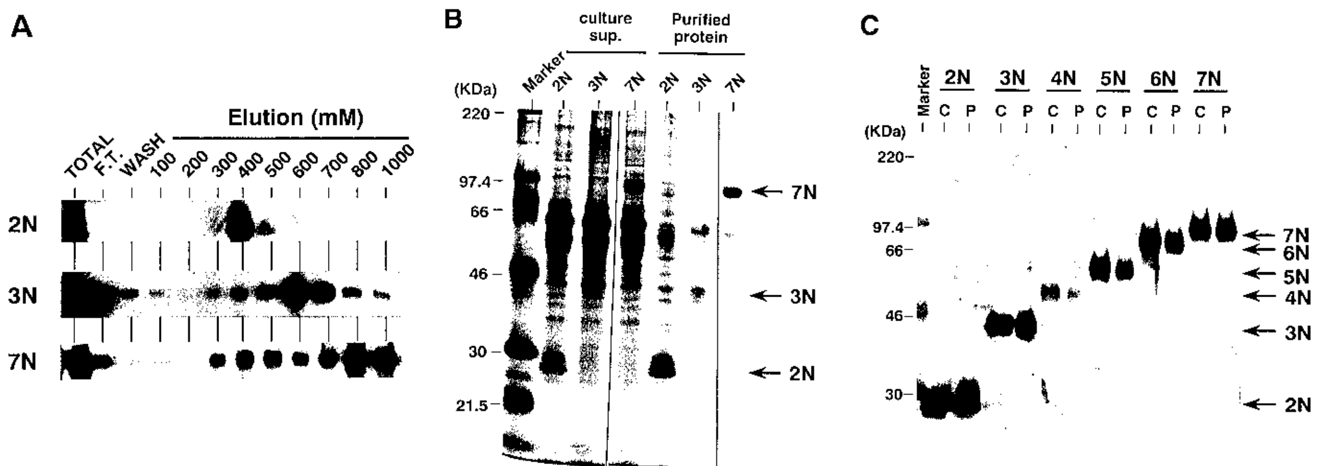

Fig. 2. Expression of sFlt-1 extracellular proteins 2N to 7N as soluble proteins from insect cells. A, Affinity chromatography of sFlt-1 proteins on a heparin column. sFlt-1 culture supernatant (1 ml) was loaded onto a HiTrap-Heparin column equilibrated with 10 m*M* sodium phosphate buffer, pH 6.2, and then washed with the same buffer. Absorbed proteins were eluted with 100 m*M* to 1 *M* NaCl-containing sodium phosphate buffer. A 1/300 volume of the eluates was western-blotted against the rabbit polyclonal antibody α-Flt-1 #2. F.T., flow-through. B, Purification of sFlt-1 extracellular proteins by using Heparin-agarose column. A 1/700 volume of total purified protein and a 1/2,000 volume of total culture supernatant were resolved by SDS/8% PAGE and bands were visualized by silver staining. The positions of standards are marked in kDa. C, Western blotting of the purified sFlt-1 proteins. The same samples were resolved by SDS/8% PAGE, transferred onto a nitrocellulose filter and immunoblotted with the rabbit polyclonal antibody α-Flt-1 #2. Proteins were visualized by chemiluminescence and autoradiography. C, crude; P, purified.

high affinity. 2N sFlt-1 showed almost undetectable binding to ^{125}I-VEGF in this plate-fixed method. To examine more directly whether or not the 2N form interacts with VEGF, 2N sFlt-1 was added as a competitor in the binding assay system between the 7N form fixed on plastic plates and soluble ^{125}I-VEGF. Although the 2N sFlt-1 was about eight- to ten-fold less efficient than 7N sFlt-1 as a competitor, it blocked VEGF binding, indicating that the 2N sFlt-1 construct could bind VEGF to some extent (Fig. 5).

The results were essentially the same for PlGF. The 3N to 7N, but not the 2N form of sFlt-1 bound to ^{125}I-PlGF with high affinity (Fig. 6). The affinity of sFlt-1 for VEGF or PlGF is summarized in Table I. The degree of affinity of the 3N to 7N sFlt-1 forms, except 2N, for these ligands, did not differ much. The 2-fold difference in K_d values for PlGF and 3-fold difference in those for VEGF might be due to conformational changes caused by binding of the sFlt-1 molecules to the plastic plates.

Cross-linking of the soluble Flt-1 with VEGF Since even the 3N sFlt-1 form showed a high binding affinity for the ligands, we examined the role of the 4th to 7th Ig region

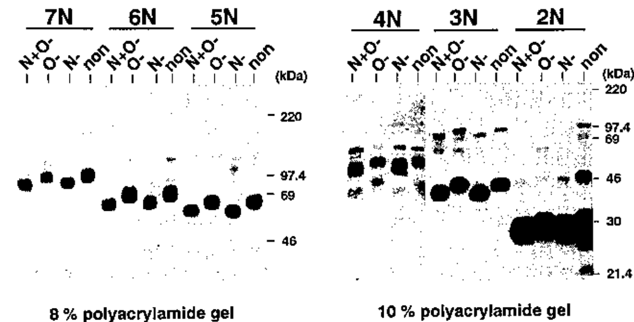

Fig. 3. Carbohydrate analysis of the sFlt-1 proteins. Purified sFlt-1 proteins (10–50 ng) were digested with N-glycosidase F and O-glycosidase. The digest was western-blotted against the rabbit polyclonal antibody, α-Flt-1#2. N-, N-glycosidase F; O-, O-glycosidase.

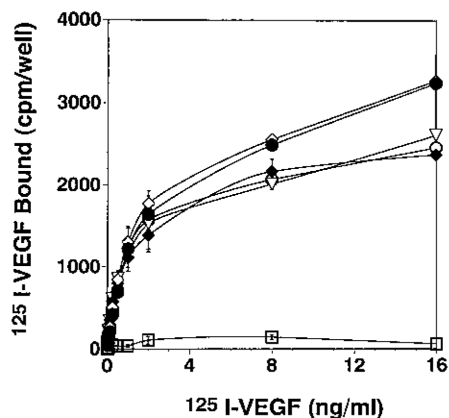

Fig. 4. Solid-phase binding assay of ^{125}I-VEGF binding to sFlt-1 proteins. Purified sFlt-1 proteins (10 ng/well) were absorbed on the surface of 96-well plates. Various amounts of ^{125}I-labeled VEGF were added to the wells and the plates were incubated for 3 h at 20°C. The wells were rinsed, then bound proteins were quantified in a gamma counter. Each point represents the average of triplicate determinations. The error bars indicate standard deviation. □, 2N; ○, 3N; ▽, 4N; ◆, 5N; ◇, 6N; ●, 7N.

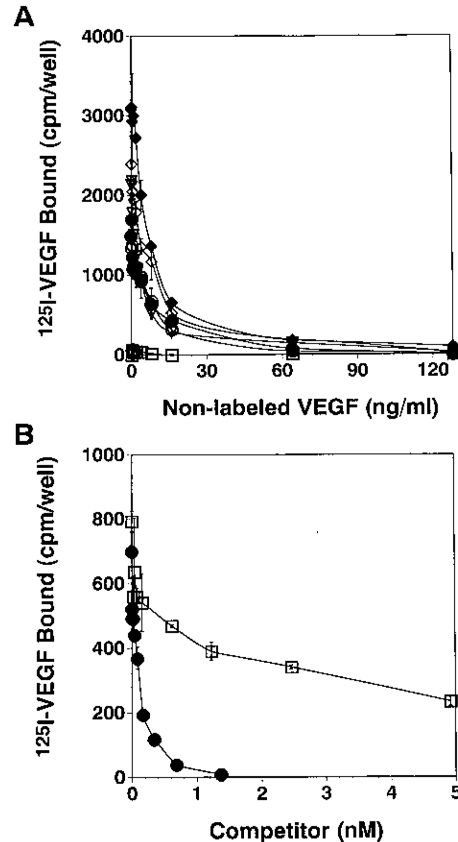

Fig. 5. Competitive solid-phase binding assay between ^{125}I-VEGF and sFlt-1 proteins. A, Inhibition of specific ^{125}I-VEGF-binding to sFlt-1 fixed on plastic wells. Binding experiments were conducted as described in the legend to Fig. 4. The fixed sFlt-1s were incubated with 1 ng/ml ^{125}I-VEGF and increasing amounts of unlabeled VEGF. B, Inhibition of ^{125}I-VEGF binding to the fixed sFlt-1 protein by 2N sFlt-1. Soluble 2N Flt-1 protein was added as a competitor, instead of unlabeled VEGF. Under these experimental conditions, neither bFGF (64 ng/ml, 3.5 nM) nor PDGF (64 ng/ml, 2 nM) showed any competitive activity to the binding of ^{125}I-VEGF to Flt-1. □, 2N; ○, 3N; ▽, 4N; ◆, 5N; ◇, 6N; ●, 7N.

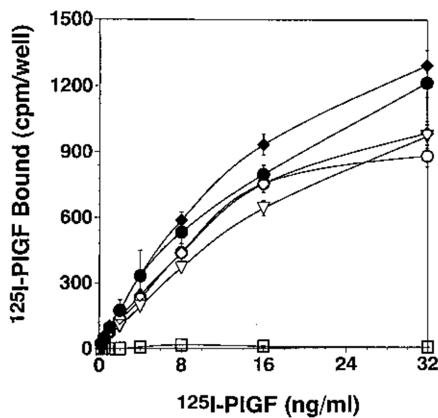

Fig. 6. ^{125}I-PlGF binding to sFlt-1 proteins. Solid-phase binding assay of ^{125}I-PlGF was conducted as described in the legend to Fig. 4. □, 2N; ○, 3N; ▽, 4N; ◆, 5N; ◇, 6N; ●, 7N.

Table I. K_d Values between sFlt-1 Molecules and $VEGF_{165}$ or $PlGF_{131}$

sFlt-1 molecules	K_d (pM)	
	VEGF	PlGF
2N	ND	ND
3N	6.5	447
4N	4.8	246
5N	5.0	253
6N	13.5	194
7N	17.0	193

ND: no detectable binding in solid-phase assay.

in ligand-receptor interaction. It is well known that dimer or oligomer formation of receptor-type tyrosine kinase is required to activate tyrosine kinase in association with the appropriate ligand.[27] Therefore, we performed cross-linking experiments using these sFlt-1 molecules, ^{125}I-VEGF and the cross-linker, *bis*(sulfosuccinimidyl)suberate (Fig. 7A). At 1 ng of sFlt-1 molecules, all except the 2N cross-linked with ^{125}I-VEGF. Furthermore, 4N to 7N, but not 3N, formed dimers.

When the amount of sFlt-1s was increased 10-fold (10 ng), the dimer formation of 4N to 7N forms significantly increased. The molar ratio of the dimer to the monomer forms was about 1 to 3 in 4N Flt-1, and 2 to 3 in 5N, 6N and 7N Flt-1 (Fig. 7A, right). However, 3N sFlt-1 did not form clear dimers. The 2N molecule formed neither monomers nor dimers under the cross-linking conditions using 0.1 or 1 ng of sFlt-1. At a higher concentration of sFlt-1 (10 ng), we detected a very faint band of the monomer.

To confirm the specificity of the cross-linking reaction, we performed competition studies using non-radiolabeled VEGF, basic FGF or PDGF. As indicated in Fig. 7B, only VEGF strongly competed with both monomer and dimer formation of 6N sFlt-1 for binding to ^{125}I-VEGF. These results suggest that the biological role of the 4th Ig to 7th Ig region is to form Flt-1 receptor dimers, and that, although the 4th Ig domain carries a dimer-forming activity, the region of the 5th to 7th domains also plays a role in increasing the dimer formation of Flt-1.

Suppression of VEGF-dependent endothelial cell growth by soluble Flt-1s The above results using the soluble forms of Flt-1 suggest that only one molecule of 3N sFlt-1 binds with high affinity to one molecule of VEGF homodimer. To test whether or not this binding of 3N to VEGF could block the biological activity of VEGF, we used an *in vitro* culture system of primary rat sinusoidal endothelial cells obtained from liver tissue by collagenase perfusion.[27] The proliferation of these endothelial cells is highly dependent on exogenously added VEGF. A 10- to 250-fold excess of 3N to 7N sFlt-1 compared with the amount of VEGF was added to the culture medium and we examined the number of cells after 4 days. As indicated in Fig. 8, 3N to 7N, but not 2N sFlt-1, suppressed endothelial cell growth in the presence of VEGF with similar efficiency. Thus, although the 3N form may bind only one side of the VEGF molecule, this short form, as well as the larger forms of sFlt-1, efficiently blocked the biological activity of VEGF.

Signal transduction from Flt-1 receptor mutants on COS cells To examine further the role of the 4N to 7N portion of Flt-1 in signal transduction, we established COS cell lines overexpressing (1) full length Flt-1, (2) the 4th Ig domain-deleted mutant of Flt-1 (del 4N Flt-1) and (3) the 4th to 7th Ig domain-deleted mutant of Flt-1 (del 4–7N Flt-1). All these constructs contain the normal transmembrane domain and the cytoplasmic region including the tyrosine kinase domain.

As shown in Fig. 9, the full-length Flt-1 showed an increase in autophosphorylation in response to VEGF, although the degree of phosphorylation was low, as indicated by the features of Flt-1. Similarly, del 4N Flt-1 also showed an increase in autophosphorylation. However, del 4–7N Flt-1 did not show a significant response to VEGF in terms of autophosphorylation levels. These results suggest that the 4th to 7th Ig region is important for the activation of Flt-1 kinase, and that it most likely contributes to the formation of receptor dimers.

A slight increase in the autophosphorylation of del 4–7N Flt-1 without VEGF might be due to spontaneous activation of tyrosine kinase owing to a conformational change after the significant deletion mutation in the extracellular domain.

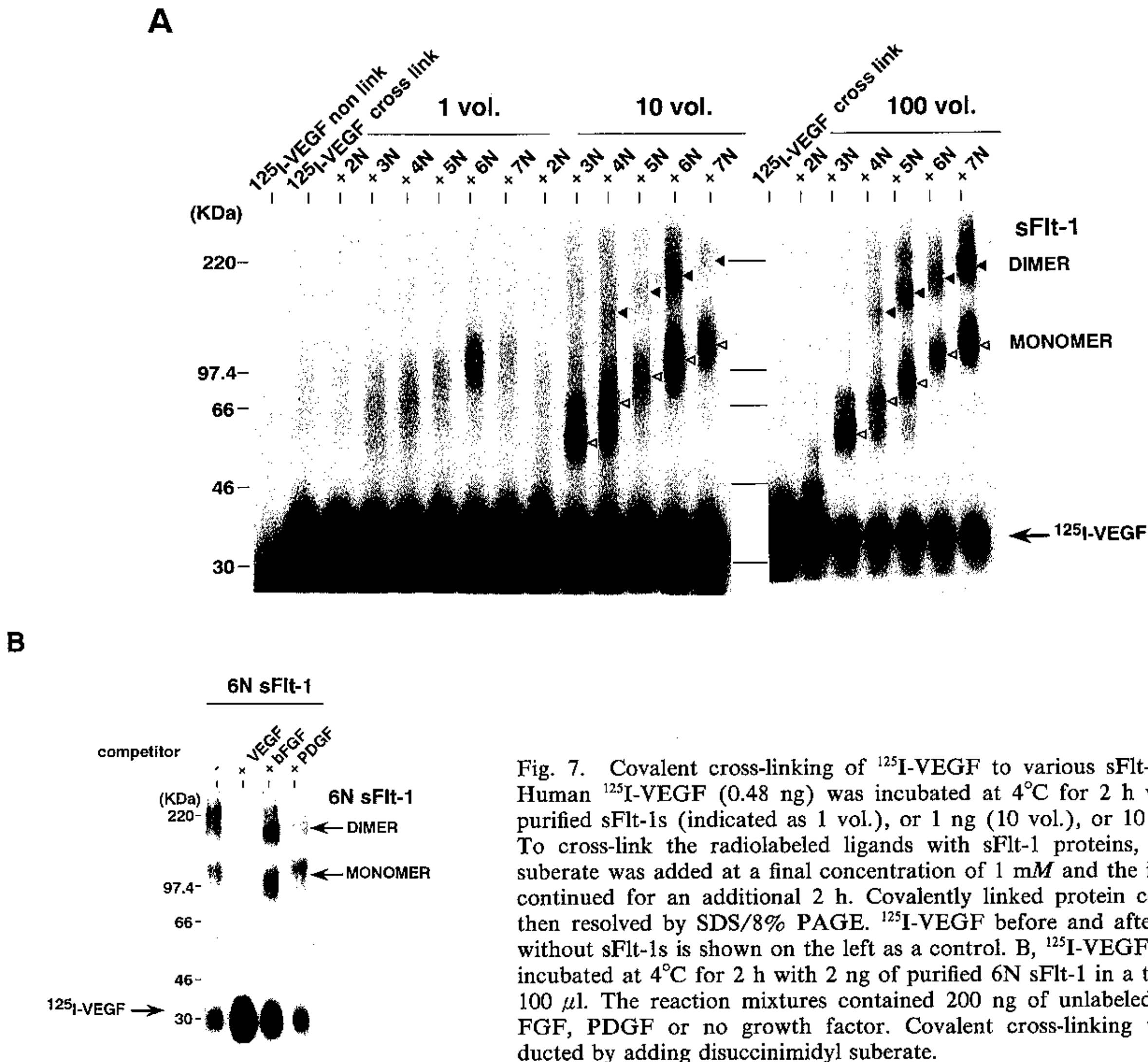

Fig. 7. Covalent cross-linking of ^{125}I-VEGF to various sFlt-1 proteins. A, Human ^{125}I-VEGF (0.48 ng) was incubated at 4°C for 2 h with 0.1 ng of purified sFlt-1s (indicated as 1 vol.), or 1 ng (10 vol.), or 10 ng (100 vol.). To cross-link the radiolabeled ligands with sFlt-1 proteins, disuccinimidyl suberate was added at a final concentration of 1 m*M* and the incubation was continued for an additional 2 h. Covalently linked protein complexes were then resolved by SDS/8% PAGE. ^{125}I-VEGF before and after cross-linking without sFlt-1s is shown on the left as a control. B, ^{125}I-VEGF (0.24 ng) was incubated at 4°C for 2 h with 2 ng of purified 6N sFlt-1 in a total volume of 100 μl. The reaction mixtures contained 200 ng of unlabeled VEGF, basic FGF, PDGF or no growth factor. Covalent cross-linking was then conducted by adding disuccinimidyl suberate.

DISCUSSION

In this study we examined the functions of the extracellular domain of the 7-Ig type tyrosine kinase receptor Flt-1 and showed that the N-terminal 340 amino acids carrying the 1st to 3rd Ig-domain and the further downstream region have different roles in receptor-mediated signal transduction. We concluded that the former is mainly utilized for high-affinity binding to the ligands and that the latter contributes to receptor dimer formation.[28]

Based on the structural relationship between the 5-Ig type receptors [CSF-1 receptor (c-Fms), stem cell factor receptor (c-Kit), PDGF receptor] and the 7-Ig type receptors [Flt-1 (VEGFR-1), KDR/Flk-1 (VEGFR-2), Flt-4], the functions of each region in the receptor molecules could be similar in these two receptor families.[6,7,9,29,30] Blechman *et al.*[31] showed that the ligand-binding domain of c-Kit could be separated into the 1st to the 3rd, the 4th and 5th Ig regions. The 1st to 3rd Ig region carries the major ligand-binding site. However, the binding affinity of this region to the ligand is significantly lower than that of the whole extracellular domain. On the other hand, the 4th Ig region is involved in dimer formation of the receptor in association with the ligand, resulting in a strong interaction between the receptor and the ligand. The function of the 5th Ig region is still not clear.

When the results obtained in this study were compared with the previous observations,[31] the Flt-1 1st to 3rd Ig

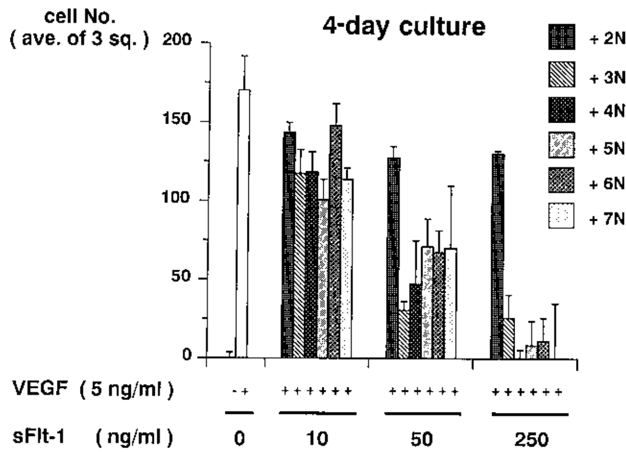

Fig. 8. Inhibition of VEGF-dependent sinusoidal endothelial cell proliferation by sFlt-1 proteins. Rat liver nonparenchymal cells were prepared as described in "Materials and Methods" and seeded in DMEM with 5% fetal calf serum in collagen-coated 24-well plates (1×10^5 cells/well). After 3 h, the culture medium was changed to the endothelial culture medium EGM-UV with 10, 50, or 250 ng/ml of sFlt-1 proteins followed by the addition of purified human VEGF. Endothelial cells in 1.0×1.5 mm rectangles at three positions were counted and the average number was plotted. The numbers of macrophage-like and epithelial cells which did not respond to VEGF were subtracted. The error bars indicate standard deviation.

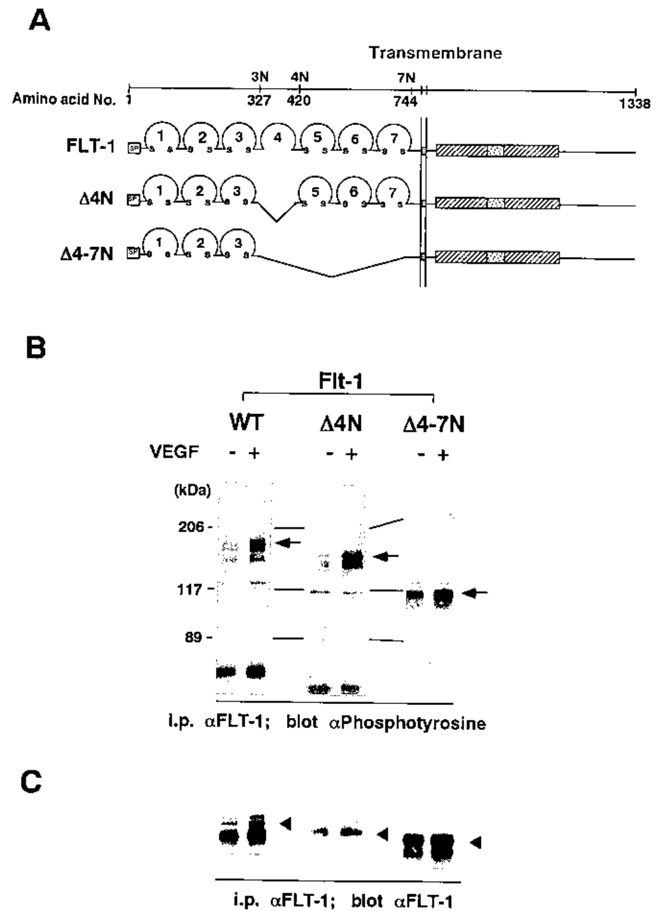

Fig. 9. Autophosphorylation of Flt-1 mutant receptors expressed on COS cells. A, Schematic representation of the Flt-1 mutant receptors. B, Decrease in autophosphorylation of the del 4–7N Flt-1 receptor in response to VEGF. COS cell clones expressing the wild-type (WT), del 4N or del 4–7N Flt-1 receptor were starved for 24 h, then stimulated with 50 ng/ml of VEGF (see "Materials and Methods"). After 5 min stimulation, cell lysates were prepared, immunoprecipitated with anti Flt-1 antiserum P1-2, separated by SDS-PAGE, then western-blotted with the anti phosphotyrosine antibodies PY20 and 4G10. C, Aliquots of the same samples shown in (A) were immunoprecipitated with anti Flt-1 antiserum P1-2, and blotted against the anti Flt-1 antiserum N2. Arrows in (B) and arrowheads in (C) correspond to the positions of mature wild-type and mutant Flt-1 proteins.

region similarly served as the major binding site for both VEGF and PlGF. However, the binding affinities between this short N-terminal region and the ligands were comparable to those between the whole Flt-1 extracellular domain and the ligands. Thus, the major difference between 3N Flt-1 and the larger 4N to 7N forms is the activity for dimer or oligomer formation of the receptors, but not the degree of affinity for the ligands.

Based on the results of the first assay system using the sFlt-1s fixed on plastic plates, it appeared that the 2N form has no binding affinity for VEGF or PlGF. However, a competition assay using the sFlt-1s without fixation clearly indicated that even the 2N form has some affinity for the ligands. This suggests that fixation of 2N Flt-1 to the plates without a linker or a tag molecule may disturb the integrity of its 3-dimensional structure necessary for binding to VEGF. Further, our preliminary experiments showed that a molecule carrying the 2N region plus a portion of the 3rd Ig domain has significantly high affinity for the ligand (Okamoto, Suzuki and Shibuya, unpublished). Thus, the high-affinity binding site(s) in the extracellular domain is considered to be localized within the intact sequence of the 1st to the 2nd Ig region and a partial sequence of the 3rd Ig region.

An interesting question is why the homodimer of VEGF binds to only one molecule of the 3N form. We have no reasonable explanation for this, but one possibility is that the binding reaction of VEGF with 3N causes a conformational change of the homodimer of VEGF. Thus, the other site of VEGF might not bind another 3N molecule with high affinity. However, if the receptor contains the 4th Ig or a further downstream sequence of

the extracellular domain of Flt-1, this region could function as a structure for dimer formation with other receptors through tight association involving two receptor molecules. Another possibility might be steric hindrance of the binding region on the opposite side of VEGF after the binding with one 3N sFlt-1 molecule.

In addition, although it appears unlikely, 3N Flt-1 may have bound to both sides of the VEGF molecule, but the cross-linking reaction itself might not have worked efficiently in this case. This association with the ligand and dimer formation of the receptors should be studied in more detail.

Concerning the dimer-forming activity in the 4th to 7th Ig domains of Flt-1, we originally expected that only the 4th Ig domain would have the activity for receptor dimerization, as in the case of c-Kit, 5-Ig receptor.[31] However, both the cross-linking experiment (Fig. 7) and the signal transduction experiment (Fig. 9) suggested that, in addition to the 4th Ig domain, the region of the 5th to 7th Ig domain also has a capacity for receptor dimerization. Although we have not yet identified in detail the second region for receptor dimerization within the 5th to 7th domains, the longer structure of the extracellular domain in the 7-Ig receptors such as Flt-1 may offer some advantage in receptor activation by utilizing multiple dimer-forming regions, when compared to the 5-Ig receptors which have only one region (4th Ig domain) for dimerization. A possible model for the association of VEGF to Flt-1 extracellular domain is shown in Fig. 10.

Davis-Smyth *et al.*[19] have recently shown that the 2nd Ig-like domain of Flt-1 binds VEGF. They achieved this by using fusion molecules between the extracellular portion of Flt-1 and human heavy chain IgG-derived carboxyl terminal peptide, as well as chimeric receptor constructs between Flt-1 and Flt-4. These systems are useful for defining the ligand-binding region that is inserted with a peptide different from the original sequence, but it is difficult to evaluate the dimer formation of the Flt-1 extracellular domain, since human IgG peptide and another intracellular domain of Flt-4 fused to the sFlt-1 may have some effect upon dimer formation. IgG molecules form dimers through disulfide bonds at the carboxyl region. Thus, the above results and the data presented here may be complementary in understanding the characteristics of the N-terminal half region of the VEGF receptor Flt-1 in terms of the intramolecular localization of the ligand-binding site and the dimer-forming region.

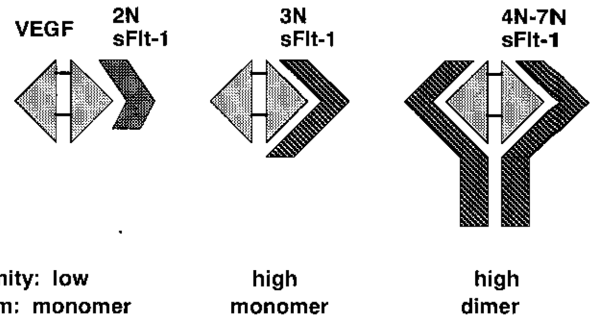

Fig. 10. A hypothetical model for the association of VEGF to Flt-1 extracellular domain.

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