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The second immunoglobulin-like domain of the VEGF tyrosine kinase receptor Flt-1 determines ligand binding and may initiate a signal transduction cascade

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Vascular endothelial growth factor (VEGF) is an angiogenic inducer that mediates its effects through two high affinity receptor tyrosine kinases, Flt-1 and KDR. Flt-1 is required for endothelial cell morphogenesis whereas KDR is involved primarily in mitogenesis. Flt-1 has an alternative ligand, placenta growth factor (PlGF). Both Flt-1 and KDR have seven immunoglobulin (Ig)-like domains in the extracellular domain. The significance and function of these domains for ligand binding and receptor activation are unknown. Here we show that deletion of the second domain of Flt-1 completely abolishes the binding of VEGF. Introduction of the second domain of KDR into an Flt-1 mutant lacking the homologous domain restored VEGF binding. However, the ligand specificity was characteristic of the KDR receptor. We then created chimeric receptors where the first three or just the second Ig-like domains of Flt-1 replaced the corresponding domains in Flt-4, a receptor that does not bind VEGF, and analyzed their ability to bind VEGF. Both swaps conferred upon Flt-4 the ability to bind VEGF with an affinity nearly identical to that of wild-type Flt-1. Furthermore, transfected cells expressing these chimeric Flt-4 receptors exhibited increased DNA synthesis in response to VEGF or PlGF. These results demonstrate that a single Ig-like domain is the major determinant for VEGF–PlGF interaction and that binding to this domain may initiate a signal transduction cascade.

Keywords: angiogenesis/*fms*-like tyrosine kinase/placenta growth factor/signal transduction/vascular endothelial growth factor

Introduction

Angiogenesis plays a major role in physiological as well as pathological conditions (Folkman, 1995). The regulation of angiogenesis is thought to occur via a balance between angiogenic inducers and inhibitors interacting through specific receptors on target cells (Mustonen and Alitalo, 1995). Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen and angiogenic factor (Ferrara, 1993). The pivotal role of VEGF in the development of the vascular system is emphasized by the recent finding that loss of a single VEGF allele results in embryonic lethality (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). Also, other studies have shown that VEGF is a key mediator of neovascularization associated with tumors and retinopathies (Kim *et al.*, 1993; Aiello *et al.*, 1994). Furthermore, VEGF administration promotes collateral vessel growth in animal models of coronary or limb ischemia (Takeshita *et al.*, 1994; Pearlman *et al.*, 1995). Consistent with the hypothesis that VEGF is an endothelial cell-specific factor, ligand autoradiography studies revealed that high affinity VEGF binding sites are localized to the vascular endothelium of large or small vessels but not to other cell types (Jakeman *et al.*, 1992, 1993). Two tyrosine kinases have been identified as receptors for VEGF. The Flt-1 (Shibuya *et al.*, 1990; de Vries *et al.*, 1992) and KDR (Terman *et al.*, 1992; Millauer *et al.*, 1993; Quinn *et al.*, 1993) proteins have been shown to bind VEGF with high affinity. The murine homolog of KDR, Flk-1, has also been identified and shares 85% sequence identity with human KDR (Matthews *et al.*, 1991). Both Flt-1 and KDR mRNAs are selectively expressed in vascular endothelial cells in both fetal and adult tissues (Millauer *et al.*, 1993; Peters *et al.*, 1993; Quinn *et al.*, 1993). Flt-1 and KDR each have seven immunoglobulin (Ig)-like domains in the extracellular domain (ECD), a single transmembrane region and a consensus tyrosine kinase sequence which is interrupted by a kinase-insert region (Shibuya *et al.*, 1990; Matthews *et al.*, 1991; Terman *et al.*, 1991; de Vries *et al.*, 1992). Flt-1 has the highest affinity for VEGF, with a K_d of ~10–20 pM (de Vries *et al.*, 1992); KDR has a lower K_d of ~100–125 pM (Terman *et al.*, 1992; Millauer *et al.*, 1993). While KDR binds only VEGF with high affinity, Flt-1 also binds placenta growth factor (PlGF) (Park *et al.*, 1994), a molecule with significant structural homology to VEGF (Maglione *et al.*, 1991). An additional member of the family of receptor tyrosine kinases (RTKs) with seven Ig-like domains in the ECD is Flt-4 (Pajusola *et al.*, 1992; Finnerty *et al.*, 1993; Galland *et al.*, 1993). Flt-4 is not a receptor for either VEGF or PlGF, but rather binds a recently identified ligand called VEGF-C or VEGF-related protein (VRP) (Joukov *et al.*, 1996; Lee *et al.*, 1996). This newly described growth factor shares sequence homology with VEGF and PlGF of 32 and 27%, respectively (Joukov *et al.*, 1996; Lee *et al.*, 1996). Like VEGF, VEGF-C/VRP can induce mitogenesis in vascular endothelial cells, albeit at a 100-fold less potency (Lee *et al.*, 1996).

The Flt-1 and KDR proteins have been shown to have different signal transduction properties (Waltenberger *et al.*, 1994; Seetharam *et al.*, 1995). While KDR undergoes strong ligand-dependent tyrosine phosphorylation in intact cells (Terman *et al.*, 1992; Millauer *et al.*, 1993; Waltenberger *et al.*, 1994), Flt-1 reveals a weak response (de Vries *et al.*, 1992; Waltenberger *et al.*, 1994; Seetharam *et al.*, 1995). Furthermore, transfected cells expressing

Fig. 1. Alignment of the ECDs of Flt-1, KDR and Flt-4. The seven Ig-like domains are shown as shaded boxed areas. Note that the amino acid numbering begins with position 1 at the first Met of the signal peptide sequence.

each receptor reveal distinctive patterns of phosphorylation of a variety of intracellular proteins in response to VEGF (Waltenberger *et al.*, 1994). Several studies have implicated KDR but not Flt-1 in VEGF-induced mitogenesis (Millauer *et al.*, 1994; Park *et al.*, 1994; Waltenberger *et al.*, 1994; Keyt *et al.*, 1996). Gene knockout studies have shed light on the role of Flt-1 versus KDR and demonstrated that both receptors are essential for the development and differentiation of embryonic vasculature (Fong *et al.*, 1995; Shalaby *et al.*, 1995). Mice null for the Flk-1/KDR gene lacked vasculogenesis and blood island formation, resulting in death *in utero* between days 8.5 and 9.5 (Shalaby *et al.*, 1995). Mouse embryos homozygous for a targeted mutation in the Flt-1 locus also died *in utero* around day 8.5 (Fong *et al.*, 1995). Endothelial cells developed in both embryonic and extra-embryonic sites but failed to organize in normal vascular channels, suggesting that Flt-1 is involved primarily in endothelial cell morphogenesis.

Several growth factors including platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and fibroblast growth factor (FGF) mediate their biological actions through receptors with tyrosine kinase activity (Ullrich and Schlessinger, 1990; Fantl *et al.*, 1993). The receptors often undergo oligomerization, following ligand stimulation, resulting in their activation (Heldin, 1995). Elucidating how growth factors activate their receptors is essential for our understanding of the sequence of events which results in transduction of cellular regulatory signals. Towards this end, we examined the functional roles of the Ig-like domains within the ECD of Flt-1. We not only demonstrate that a single domain is the major determinant for VEGF/PlGF binding, but also that ligand interaction with this domain, even in the context of an ECD of a foreign receptor, is able to trigger a signal transduction cascade.

Results

The second immunoglobulin-like domain determines ligand binding and specificity of the Flt-1 receptor

The boundaries of the seven immunoglobulin-like domains in the ECDs of Flt-1, KDR and Flt-4 were first defined based on conserved cysteines, predicted β-strands and other sequence considerations (Williams and Barclay, 1988; Finnerty *et al.*, 1993). Figure 1 displays the alignment of the ECDs of the receptors and the Ig-like domains defined within this region for each.

A set of Flt-1 receptor Ig-like domain deletions and chimeric variants was constructed (Figure 2). Previously it has been shown that the ECD of Flt-1 or KDR fused to an IgG heavy chain was capable of binding VEGF with the same K_d as the full-length membrane-bound receptors (Park *et al.*, 1994). Therefore, dimerization induced by the presence of the IgG does not alter the ligand binding ability of the ECD of the VEGF receptors. Oligonucleotide-directed mutagenesis was employed to specifically 'loop-out' each Ig-like domain. Conditioned media from CEN4 cells transiently expressing the domain deletion mutants was used in VEGF binding assays. Analysis of the Flt-1 domain deletion–IgG soluble receptors revealed that removal of domain 2 completely abolished the receptor's ability to bind VEGF (Figure 3A). In contrast,

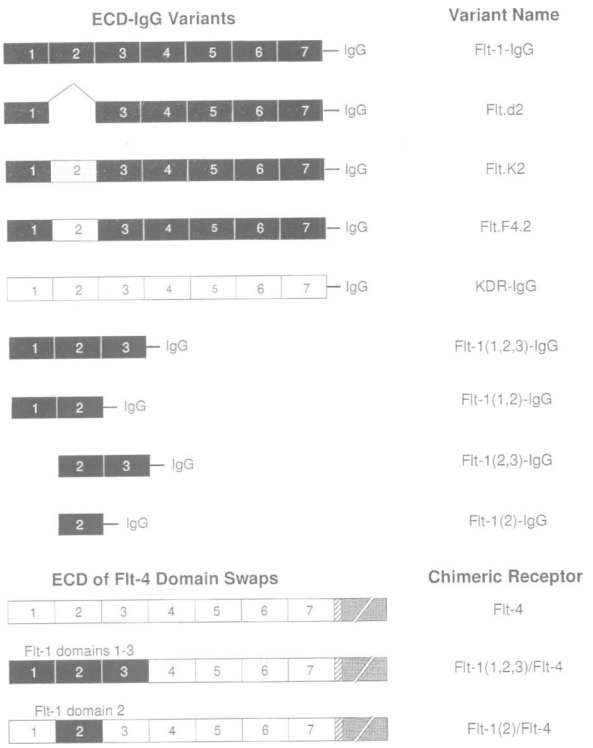

Fig. 2. Schematic representation of several constructs utilized in this study. The first set depicts the ECD regions fused to Fc–IgG. A domain deletion construct was created for each Ig-like domain of Flt-1, although only the second domain deletion variant is represented here. Solid black boxes represent Flt-1 domains, gray boxes depict KDR domains and open boxes Flt-4 domains. The latter group illustrates the Flt-4 wild-type and chimeric receptors. The Ig-like domains are represented as above, the transmembrane region is denoted by the diagonal stripe area and the cytoplasmic portion is shown as the broken stippled rectangle. None of the schematics are drawn to scale.

deletion of other domains was associated with a reduction in the amount of VEGF bound to the receptor (Figure 3A). [^{125}I]VEGF binding to such deletion mutants could be fully displaced by cold VEGF, at concentrations similar to those required for the wild-type receptor, suggesting that the observed reduction in binding was due, at least in part, to incorrect folding of the mutant proteins. Interestingly, PlGF was able to compete with [^{125}I]VEGF for binding to these deletion mutants, confirming that the binding sites of VEGF and PlGF are largely overlapping (data not shown).

Replacing the second domain of Flt-1 with that of KDR (Flt.K2) restored the mutant's ability to bind VEGF (Figure 3B). In contrast, introduction of domain 2 of Flt-4 (Flt.F4.2) did not restore binding. We next attempted to determine whether the swap of domain 2 of KDR conferred on Flt-1 the ligand specificity of KDR, namely the ability to bind VEGF but not PlGF (Park *et al.*, 1994). Accordingly, PlGF was not able to displace [^{125}I]VEGF bound to Flt.K2, whereas it efficiently competed with VEGF for binding to wild-type Flt-1 (Figure 3C). Therefore, VEGF binding determinants are localized to the second Ig-like domain of both VEGF receptors.

To assess directly whether the primary determinants for VEGF/PlGF binding in Flt-1 reside in domain 2 alone or require the contribution of additional sequences within the flanking domains, Flt-1/Flt-4 chimeric receptors were

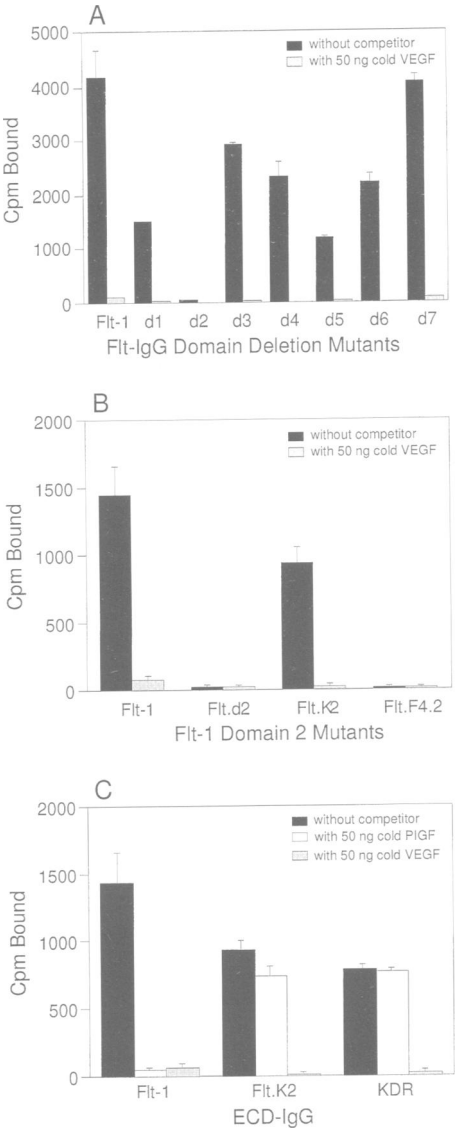

Fig. 3. Domain 2 is the primary domain responsible for ligand binding in VEGF receptors. In (**A**), Flt-1–IgG deletion variants (5 ng per reaction) were tested for their ability to bind [^{125}I]$VEGF_{165}$ in the absence (solid bars) or presence of 50 ng cold $VEGF_{165}$ (gray bars) as described in Materials and methods. Deletion of the second domain completely abolishes the binding of VEGF. The second domain of either KDR or Flt-4 was cloned into the Flt-1 domain 2 deletion construct to produce domain 2 'swap' mutants (**B**). Replacing domain 2 of Flt-1 with the homologous domain of KDR re-established VEGF binding. In contrast, introduction of Flt-4 domain 2 failed to promote interaction with VEGF. In addition, $PlGF_{152}$ (white bars) could not displace $VEGF_{165}$ bound to Flt.K2, a pattern characteristic of the KDR but not the Flt-1 receptor (**C**). These experiments were performed in duplicate.

generated. We swapped either the first three domains or the second domain of Flt-1 into Flt-4 to create Flt-1(1,2,3)/Flt-4 or Flt-1(2)/Flt-4, respectively (Figure 2). NIH 3T3 clones stably expressing either chimeric receptor were created. The binding affinity of the chimeric receptors for VEGF was then determined by ligand displacement in cell binding assays (Figure 4A and B). Scatchard analysis indicated that both chimeric receptors bound VEGF with a high affinity (K_d of 11.3 and 16.3 pM, respectively) (Figure 4, insets). These values are within the range reported for the full-length Flt-1 receptor. In addition,

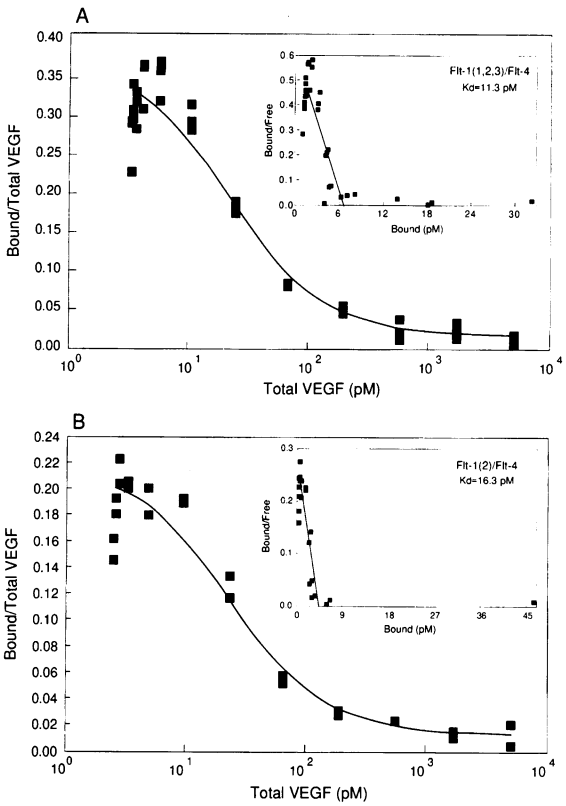

Fig. 4. Competitve displacement of [^{125}I]VEGF$_{165}$ from Flt-1(1,2,3)/Flt-4 and Flt-1(2)/Flt-4 chimeric receptors with cold VEGF$_{165}$. Cells stably expressing Flt-1(1,2,3)/Flt-4 (**A**) or Flt-1(2)/Flt-4 (**B**) were analyzed in cell binding assays to determine the K_d for VEGF by titrating a trace amount of [^{125}I]VEGF$_{165}$ with increasing amounts of cold VEGF$_{165}$. Data plotted by the method of Scatchard are shown in the insets. K_d values for Flt-1(1,2,3)/Flt-4 and Flt-1(2)/Flt-4 are 11.3 ± 2.6 and 16.3 ± 3.8 pM, respectively. Each experiment was performed in triplicate.

PlGF could compete for [^{125}I]VEGF binding to both chimeric receptors (data not shown).

VEGF-induced autophosphorylation of Flt-1/Flt-4 receptor chimeras and induction of DNA synthesis in transfected cells

To examine the possibility that VEGF binding to domain 2 resulted in the induction of a signal transduction response, 293 cells transiently expressing Flt-1(2)/Flt-4 were treated with VEGF or VEGF-C/VRP. Immunoprecipitation of the Flt-1(2)/Flt-4 chimeric receptor and subsequent Western blotting with an anti-phosphotyrosine antibody showed that VEGF addition stimulated tyrosine autophosphorylation (Figure 5). VEGF-C did not stimulate this phosphorylation appreciably. Furthermore, increased tyrosine phosphorylation was observed when cells expressing Flt-1(2)/Flt-4 were treated with PlGF (data not shown).

We next sought to determine whether tyrosine phosphorylation in such chimeric receptors resulted in a mitogenic signal. Chimeric Flt-4 receptors possessing the colony-stimulating factor (CSF) receptor ECD fused to the transmembrane and cytosolic portion of Flt-4 displayed ligand-dependent tyrosine phosphorylation and induction of DNA synthesis (Pajusola *et al.*, 1994). Thus, we proceeded to perform thymidine incorporation assays in

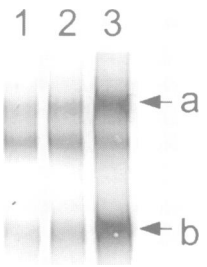

Fig. 5. Effect of VEGF$_{165}$ on tyrosine auto-phosphorylation of the Flt-1(2)/Flt-4 chimeric receptor. 293 cells transiently expressing the Flt-1(2)/Flt-4 receptor chimera were either not stimulated (lane 1) or stimulated with 400 ng/ml VEGF-C (lane 2) or 50 ng/ml VEGF$_{165}$ (lane 3) as described in Materials and methods. Following immunoprecipitation of the chimeric receptor, electrophoresis on SDS–PAGE and transfer to nitrocelluse, the samples were probed with an anti-phosphotyrosine monoclonal antibody. Stimulation with VEGF induced an increase in tyrosine auto-phosphorylation of the Flt-1(2)/Flt-4 chimeric receptor (arrow a). The lower band (arrow b) is consistent with a proteolyically cleaved product of Flt-4 resulting from a disruption within domain 5 in the ECD but possessing the cytoplasmic region (Pajusola *et al.*, 1994). The molecular weights of these bands are respectively 180 and 120 kDa.

NIH 3T3 cells stably expressing the Flt-1/Flt-4 chimeras. As shown in Figure 6A and B, both clones exhibited a 4-fold increase in thymidine incorporation in response to either VEGF or PlGF. The stimulation by either ligand was concentration dependent. The ED$_{50}$ for VEGF was estimated to be ~50 ng/ml. Both chimeric transfectants showed nearly identical responses, suggesting that VEGF-dependent signal transduction in Flt-1(2)/Flt-4 receptor was as effective as that originating in the chimeric receptor containing the first three Flt-1 domains. These findings further indicate that the primary determinants for VEGF/PlGF binding in Flt-1 reside within domain 2. Addition of VEGF-C/VRP up to 4 μg/ml did not induce any increase in thymidine incorporation in cells expressing either chimeric receptor. In contrast, VEGF-C, tested at 4 μg/ml, induced a marked increase in thymidine incorporation in NIH 3T3 cells expressing wild-type Flt-4, while VEGF or PlGF had no effect (Figure 6C).

The flanking domains are required for full VEGF binding in the isolated domain 2

We next asked the question whether or not the second domain of Flt-1 was sufficient for VEGF binding when isolated from the context of a full-length ECD. A series of truncated receptors consisting of domain 2 in the presence or absence of its flanking domains was generated. All constructs were created as IgG fusions. As shown in Figure 7A, domain 2 alone was incapable of binding VEGF, as was the second domain combined with domain 1; the combination of domains 2 and 3 demonstrated minimal binding. In contrast, the fusion protein consisting of the first three domains of Flt-1 [Flt-1 (1,2,3)–IgG] bound VEGF with an affinity and capacity essentially identical to those of full-length Flt-1–IgG (Figure 7B).

Discussion

A considerable amount of evidence has established the importance of VEGF and its two tyrosine kinase receptors in various steps of vasculogenesis and angiogenesis

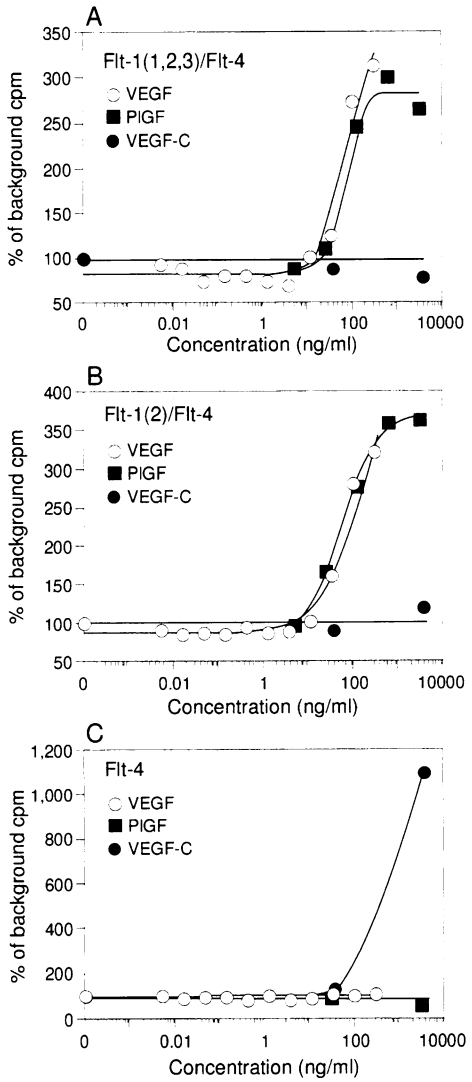

Fig. 6. Incorporation of [^{3}H]thymidine in cells expressing Flt-1(1,2,3)/Flt-4 or Flt-1(2)/Flt-4. Cells stably expressing either Flt-1(1,2,3)/Flt-4 (**A**), Flt-1(2)/Flt-4 (**B**) or Flt-4 (**C**) were stimulated with $VEGF_{165}$ (○), $PlGF_{152}$ (■) or VEGF-C (●) and DNA synthesis was measured by [^{3}H]thymidine uptake as described in Materials and methods. Ligand-induced stimulation was assessed as the percentage counts per min in non-stimulated cells. Cells expressing Flt-1(1,2,3)/Flt-4 and Flt-1(2)/Flt-4 responded to $VEGF_{165}$ or $PlGF_{152}$, but not VEGF-C. The converse was observed in Flt-4 wild-type expressing cells. Each assay was performed in triplicate.

(Ferrara, 1993; Mustonen and Alitalo, 1995; Shibuya, 1995). Here, the Ig-like domain in the ECD of Flt-1 responsible for specific ligand recognition was identified by constructing and analyzing a variety of receptor variants. These included individual domain deletions, as well as chimeras in which domains of either KDR or Flt-4 were exchanged for the homologous sequences from Flt-1. Our results demonstrate that the second Ig-like domain of Flt-1 contains critical determinants required for the interaction with both VEGF and PlGF.

Analysis of isolated Flt-1 domain–IgGs reveals that domain 2, standing alone, is incapable of binding VEGF; full binding requires the additional presence of both flanking domains, 1 and 3. This situation is unlike that described for the *trk* receptor where a single domain, expressed as an IgG fusion protein, demonstrates full neurotrophin binding (Urfer *et al.*, 1995). However, the *trk* family of receptors has only two Ig-like domains in the ECD, in addition to a leucine-rich motif and two cysteine clusters (Schneider and Schweiger, 1991). Our results emphasize a different type of interaction and indicate that the first and third Ig-like domains of Flt-1, and possibly other RTKs with multiple Ig-like domains, play an important role in maintaining the binding site in a spatial conformation compatible with ligand binding. That such flanking domains do not contribute major binding determinants, however, is indicated by the finding that both Flt-1(2)/Flt-4 and Flt-1(1,2,3)/Flt-4 bind VEGF with essentially the same affinity as wild-type Flt-1 receptor. Thus, domains 1 and 3 of Flt-1 can be substituted by the homologous domains of Flt-4, which display respectively 25 and 32% identity, without any marked influence on domain 2-mediated VEGF binding and subsequent receptor activation.

Although the emphasis of the present study is on the identification of binding elements in the Flt-1 receptor, the observation that the second domain of KDR restored VEGF, but not PlGF binding, in the Flt-1 mutant lacking the homologous domain would indicate that there are, likewise, specific binding determinants within the second domain of KDR. However, our unpublished results suggest that additional elements are required for full ligand binding in KDR. Unlike Flt-1 domain 2, the second Ig-like domain of KDR when swapped into Flt-4 was able to confer on this receptor only a modest degree of VEGF binding. Our initial analysis indicates that flanking domain sequences contribute to VEGF binding in the KDR receptor. In this context, a recent mutational analysis identifying the receptor binding determinants located within VEGF suggests that the growth factor may interact differently with each of its receptors (Keyt *et al.*, 1996). Furthermore, the exchange of Flt-4 domain 2 for that of Flt-1 rendered Flt-4 non-responsive to its natural ligand, VEGF-C, in the thymidine incorporation assay, suggesting that domain 2 is also critical for binding in the Flt-4 receptor. Therefore, it appears that the localization of at least some determinants for binding and ligand specificity within the second Ig-like domain is a common feature of subclass III RTKs with seven Ig-like domains. To examine further similarities and differences in binding requirements among these RTKs, additional studies concerning ligand interactions with KDR and Flt-4 are clearly required.

PDGF receptors belong to the same subclass III of RTKs as the VEGF receptors (Yarden and Ullrich, 1988). They have five rather than seven Ig-like domains in their ECDs. β-PDGFR binds PDGF-BB, whereas the α-PDGFR binds all three forms of PDGF. The ligand binding determinants have been mapped within the first three domains for both forms of the receptors, although to date a single domain of a PDGFR that is critical for ligand binding has not been identified (Heidaran *et al.*, 1990, 1995). Interestingly, the first domain in the α-PDGFR plays an important role in orienting the binding determinants for PDGF-AA in domains 2 and 3 (Mahadevan *et al.*, 1995). However, the determinants for binding PDGF-BB to the α-PDGFR are not influenced by the presence or absence of domain 1 (Mahadevan *et al.*, 1995). In contrast, in Flt-1, domains 1 or 3 do not appear to be differentially required for high affinity interaction with its two ligands,

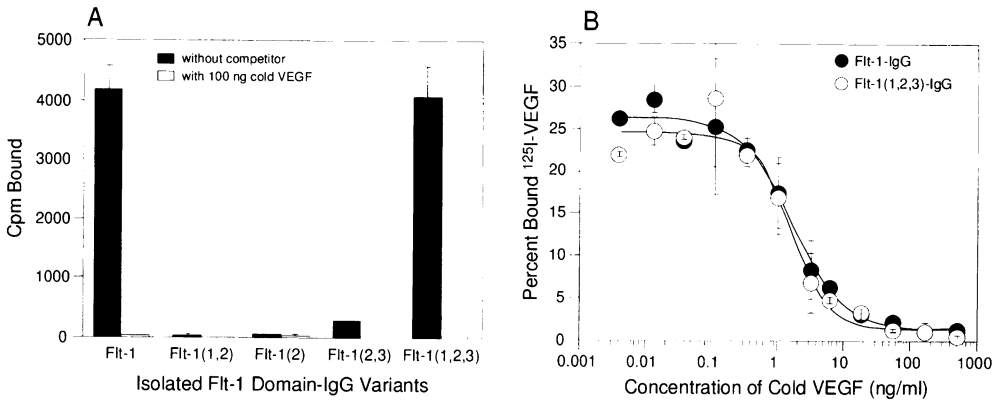

Fig. 7. Domains 1 and 3 are necessary for full VEGF binding by isolated domain 2 constructs. Truncated Fc–IgG fusion constructs of Flt-1 domain 2 [Flt-1(2)], domains 1 and 2 [Flt-1(1,2)], domains 2 and 3 [Flt-1(2,3)] and domains 1, 2 and 3 [Flt-1(1,2,3)] were tested for their ability to bind [^{125}I]$VEGF_{165}$ (**A**) as described previously; 4.5 ng of each fusion protein was used per reaction. Flt-1(1,2,3)–IgG was the only species which displayed substantial VEGF binding. Comparing competitive displacement curves of [^{125}I]$VEGF_{165}$ from either Flt-1(1,2,3)–IgG (○) or Flt-1–IgG (●) with cold $VEGF_{165}$ (**B**) attests to the nearly identical IC_{50} concentrations of 1.56 ± 0.32 and 1.58 ± 0.25 ng/ml, respectively. The assays were performed in duplicate.

VEGF and PlGF. Alanine scanning mutagenesis analysis of the first three domains of Flt-1 should allow us to define further the interaction of Flt-1 with VEGF versus PlGF.

The phenotype of Flt-1 null embryos points to a fundamental role played by this receptor in the assembly and organization of endothelial cells in tubes (Fong *et al.*, 1995). Furthermore, monocytes, expressing Flt-1 but not KDR, exhibit VEGF- or PlGF-induced chemotaxis which can be inhibited by protein kinase inhibitors (Barleon *et al.*, 1996). To date, however, the elucidation of the signal transduction mechanisms of the Flt-1 receptor has proved difficult. The inability to detect a strong ligand-dependent tyrosine phosphorylation has been a contributory factor. The Flt-1/Flt-4 chimeras are valuable tools for analyzing the influence of ECD components on the initiation and transmission of a signal transduction cascade in the Flt-1 receptor, by providing a convenient, detectable endpoint activity. The chimeric Flt-1(2)/Flt-4 receptor not only bound VEGF and PlGF, but also resulted in tyrosine autophosphorylation upon ligand stimulation. Likewise, NIH 3T3 cells stably expressing the Flt-1(2)/Flt-4 chimeric receptor experienced an induction of DNA synthesis upon stimulation by VEGF or PlGF. These findings indicate that the second Ig-like domain of Flt-1 is not only sufficient for high affinity VEGF/PlGF binding but, upon binding the ligand, is capable of initiating a signal transduction cascade.

An important question is what role do the remaining domains play in the ligand-induced signal transduction? One possible function is to provide a site, distinct from the binding site, which can permit and stabilize dimerization of the receptors. Such a role has been demonstrated for the fourth Ig-like domain in the Kit receptor, a subclass III RTK with five Ig-like domains in its ECD (Blechman *et al.*, 1995). Utilizing the series of deletions and chimeric variants, we presently are elucidating the possible roles played by the other domains in ligand-induced signal transduction.

Full-length Flt-1–IgG is a potent inhibitor of VEGF action *in vitro* and it has been shown to suppress retinal neovascularization when injected intraocularly in a mouse model of retinopathy of prematurity (Aiello *et al.*, 1995). However, due to low stability, Flt-1–IgG is not equally effective when administered systemically in other *in vivo* models (our unpublished observations). We are examining the possibility that a truncated version such as Flt-1(1,2,3)–IgG may have improved stability and bioavailability, which could translate into greater effectiveness as an inhibitor of VEGF action *in vivo*.

Materials and methods

Construction of receptor–IgG domain deletions and variants

cDNAs encoding fusion proteins consisting of the extracellular domains of human VEGF receptors, Flt-1 or KDR, and amino acids 216–443 of the human heavy chain IgGσ1(denoted –IgG) were cloned in the pHEBO23 vector (Park *et al.*, 1994). Oligo-directed mutagenesis was performed according to the method of Kunkle (1985). Following transformation into *Escherichia coli* strain XL-1 Blue, colonies were first screened via restriction digestion for the presence of newly created restriction sites (see below) and subsequently the coding region was sequenced using the Sequenase version 2.0 kit (US Biochemical Corp.). Double-stranded DNA for each selected clone was prepared using the QIAGEN DNA purification kit (Qiagen Inc.) and used for transfection into CEN4 cells.

Once the boundary positions of the Ig-like domains located within the ECD of Flt-1 were defined, oligonucleotides were designed which would remove a particular domain and introduce flanking in-frame restriction sites. The following is a description of the oligonucleotides used: underlined nucleotides distinguish the location of the restriction sites created. For the deletion of domain 1 of Flt-1 (Flt.d1), the oligonucleotide 5′-AAAATTAAAAGATCCAGATCTGAGTATCTATA-TATTTATTAGTGATACCGGTAGACCTTTT-3′ was used to loop out amino acids L36–A123 and introduce a *Bgl*II site and *Age*I site at the 5′ and 3′ ends of the domain, respectively. The *Bgl*II restriction site changed E33 to D. The oligonucleotide used to create the domain 2 deletion mutant (Flt-1.d2) was 5′-GAAGGAAACAGAAGGCGCCA-TCTATATATTTATTCGAGGTACCAATACAATCATAG-3′, effectively removing amino acids S129–H223. The creation of *Kas*I and *Kpn*I restriction sites caused amino acid changes of S122 to G and Q225 to G to occur, respectively. Deletion of the third domain removed amino acids N227–S325 (Flt.d3) using the oligonucleotide 5′-CAAACTATCT-CACACATAGATCTACCGTGCATATATATGATACCGGTTTCATCAC-TGTGAAAC-3′. Amino acids Q225, K331 and A332 were changed to S, T and G, respectively to accommodate the insertion of *Bgl*II and *Age*I restriction sites. The oligonucleotide 5′-GTTAACACCTCAGTGCAC-GTGTATGATGTCAATGTGAAACCCCAGATCTACGAAAAGGCCG-TGTC-3′ was used to loop out amino acids K331–I423 or domain 4 (Flt.d4). The amino acid change resulting from the generation of a *Bbr*PI

restriction site was I328 to V. Generating a *Bgl*II restriction site at the 3′ end did not alter any amino acids. Deleting domain 5 (Flt.d5) amino acids K427–S549 was achieved utilizing the oligonucleotide 5′-AAACCTCACTGCCACGCTAGCTGTCAATGTGTTTTATATCACAGATCTGCCAAATGGGTTTCAT-3′. Creating an *Nhe*I restriction site at the 5′ end mutated I423 to A; amino acid V555 was substituted by L during the insertion of the *Bgl*II site in the 3′ end. To generate the domain 6 deletion mutant (Flt.d6), the oligonucleotide 5′-GTGGGAAGAAACATAAGCTTTGTATACATTACAATCAGATCTCAGGAAGCACCATAC-3′ excised the amino acids T553–E652. Generating the *Bst*1107I restriction site at the 5′ end changed amino acids Y551 and I552 to V and Y, respectively; amino acid D657 was substituted by S during the formation of the *Bgl*II restriction site at the 3′ end. The last domain to be deleted, domain 7 (Flt.d7) removed amino acids Q658–Y745 while adding restriction sites *Bsi*WI and *Kpn*I 5′ and 3′, respectively. The oligonucleotide, 5′-CCAGAAGAAAGAAATTACCGTACGAGATCTCACTGTTCAAGGTACCTCGGACAAGTCTAAT-3′, did cause an amino acid substitution at I655 into V. In order to construct chimeric receptors, the desired domain fragment of either KDR or Flt-4 was amplified using PCR primers which contained the same flanking restriction sites in-frame as were created during the mutagenesis. Cutting both the deletion mutant plasmid and the PCR fragment with the restriction enzymes and subsequent ligation of the fragments yielded plasmids coding for the desired ECD–IgG chimeras.

Specific domain–IgG constructs were created by amplifying the specific domains desired, using PCR primers containing restriction sites (*Cla*I and *Bst*BI, 5′ and 3′, respectively) which provided the in-frame sites to clone into the 5′ end of the IgGσ1 heavy chain cDNA plasmid (Capon *et al.*, 1989). For Flt-1(1,2), amino acids M1–Q224 were amplified using oligonucleotides 5′-CAGGTCAATCATCGATGGTCAGCTACTGGGACACC-3′ (Flt.sp.*Cla*I) and 5′-GGTCAACTATTTCGAATTGTCGATGTGTGAG ATAG-3′ (Flt.2C.*Bst*BI). Using Flt.d1 as a template, the same two oligonucleotides were used to generate Flt-1(2). Flt-1(2, 3) was generated by amplifying Flt.d1 with the Flt.sp.*Cla*I oligonucleotide and another oligonucleotide 5′-GGTCAACTATTTCGAATATATGCACTGAGGTGTTAAC-3′ (Flt.3C.*Bst*BI) which includes the coding sequence through to I328. Amplifying Flt-1 domains 1–3 [Flt-1(1,2,3)] was accomplished using primers Flt.sp.*Cla*I and Flt.3C.*Bst*BI and Flt-1–IgG as the template. The entire domain–IgG coding sequence was then subcloned into pHEBO23 at the *Cla*I and *Not*I sites. The authenticity of all constructs used in this study was confirmed by sequence analysis.

Expression of receptors and binding assays using soluble receptors

Plasmid DNA coding for the Flt-1–IgG variants was introduced by calcium phosphate precipitation into CEN4 cells, a derivative of the human 293 cell line that expresses the Epstein–Barr virus nuclear antigen-1, required for episomal replication of the pHEBO23 vector (Su *et al.*, 1991). At 48 h post-transfection, conditioned media were collected and the concentration of soluble receptor determined by human Fc enzyme-linked immunosorbent assay (ELISA). Binding assays were performed in 96-well breakaway immunoabsorbent assay plates (Nunc) coated overnight at 4°C with 2 μg/ml affinity-purified goat anti-human IgG (Organon-Teknika) in 50 mM Na_2CO_3, pH 9.6. Plates were blocked for 1 h with 10% fetal bovine serum (FBS) in phosphate-buffered saline (PBS; buffer B). After removal of the blocking buffer, 100 μl of a binding cocktail was added to each well. Binding cocktails consisted of a given amount of IgG fusion protein, [^{125}I]$VEGF_{165}$ (<9000 c.p.m./well), and cold competitor where indicated. Unless otherwise indicated, 1 ng of receptor–IgG was used per binding reaction. CHO-derived $rhVEGF_{165}$ was iodinated by the chloramine T method as previously described (Keyt *et al.*, 1996). The specific activity of the iodinated VEGF was 5.69×10^7 c.p.m./μg. Either $rhVEGF_{165}$ or $rhPlGF_{152}$ was used as cold competitor (Park *et al.*, 1994). The binding cocktails were assembled and allowed to equilibrate overnight at 4°C prior to loading into the coated wells. Incubation in the coated wells proceeded for 4 h at room temperature, followed by several washes with buffer B. Binding was determined by counting individual wells in a gamma counter. Each experiment was performed in duplicate or triplicate. Data was analyzed using a four-parameter non-linear curve fitting program (Kalidagraph, Abelbeck Software).

Construction of full-length Flt-4 chimeric receptors

The plasmid coding for human Flt-4 (Lee *et al.*, 1996) was subjected to oligo-directed mutagenesis to create in-frame restriction sites located at the beginning of domain 1 (*Afl*II), the end of domain 1/beginning of domain 2 (*Nhe*I), the end of domain 2/beginning of domain 3 (*Bsi*WI) and the end of domain 3 (*Mlu*I). Then Flt-1 domains 1–3 or domain 2 alone were amplified using PCR primers that possessed the same in-frame restriction sites. Cloning the Flt-1 PCR products into the mutagenized Flt-4 resulted in Flt-1/Flt-4 chimeric receptor plasmids. The Flt-4 sequence coding for amino acids N33–E324 was replaced by the Flt-1 sequence corresponding to amino acids S35–S325. In addition, an amino acid change I325R in Flt-4 occurred as a result of the creation of the cloning sites. The final plasmid encoded a Flt-1(1,2,3)/Flt-4 chimeric receptor that possessed the first three Ig-like domains of Flt-1 swapped into the Flt-4 receptor. The other chimeric receptor variant was created by swapping Flt-1 amino acids I124–R224 for Flt-4 amino acids S128–I224 and changing Flt-4 amino acids N33S, I326R and adding a T36 as a result of the cloning strategy. This created a construct encoding Flt-1(2)/Flt-4 receptor chimera where the second domain of Flt-1 replaces that of Flt-4.

Tyrosine phosphorylation and cell binding assays

293 cells were transfected with plasmids encoding chimeric receptors via DEAE–dextran and transiently expressing cells were analyzed for ligand-induced tyrosine phosphorylation 60–72 h post-transfection. Tyrosine phosphorylation assays were performed essentially as described (Park *et al.*, 1994). In brief, transfected cells were deprived of serum 16–18 h prior to stimulation. Cells were stimulated with either rhVEGF-C/VRP (Lee *et al.*, 1996) at a concentration of 400 ng/ml or $rhVEGF_{165}$ at 50 ng/ml for 15 min at 37°C. Following removal of the stimulation media, the cells were lysed. The lysate was cleared of cell debris and the receptors were immunoprecipitated using a polyclonal antibody directed against the ECD of Flt-4 (Lee *et al.*, 1996). The immunoprecipitates were subjected to Western gel/blot analysis using the 4G10 anti-phosphotyrosine monoclonal antibody (UBI, Lake Placid, NY). Immunoreactive bands were visualized with an ABC kit according to the manufacturer's directions (Vector Laboratories).

To establish stable cell lines, each of the chimeric constructs was co-transfected with a plasmid containing the neomycin resistance gene via calcium phosphate precipitation into NIH 3T3 cells. Clones proliferating in the presence of G418 were screened for their ability to bind VEGF. Clones expressing either Flt-1(1,2,3)/Flt-4 or Flt-1(2)/Flt-4 chimeras were analyzed in a cell binding assay to determine the K_d for VEGF by titrating a trace amount of [^{125}I]$VEGF_{165}$ (~5000 c.p.m./ml final) with increasing amounts of cold $VEGF_{165}$ (see above for specific activity). First the adherent cells were washed with cold binding buffer C (DMEM/F12 media with 0.2% bovine serum albumin and 25 mM HEPES, pH 7.4), then [^{125}I]$VEGF_{165}$ and the cold competitor, each in 0.5 ml of buffer C, were added simultaneously. The cells were then placed at 4°C for 4 h. After aspirating off the binding buffer, the cells were washed with cold phosphate-buffered saline (PBS) and then twice with cold PBS containing 2 M NaCl. Finally, the cells were lysed with 0.25 M NaOH and the entire lysate was counted in the gamma counter. Results were analyzed for the possibility of either one- or two-site curve fits. The data consistently supported the presence of a single binding site. The K_ds were calculated using the Scatchard analysis program New Ligand (Genentech, Inc.).

Thymidine incorporation assays

NIH 3T3 cells stably expressing either Flt-1(1–3)/Flt-4 or Flt-1(2)/Flt-4 were plated in 12-well format at 50 000 cells/well in low glucose DMEM media containing 10% FBS, 100 U/ml penicillin–streptomycin (Gibco BRL), 2 mM glutamine, 2.5 μg/ml fungizone (Gibco BRL) and 200 μg/ml G418 (Gibco BRL). Following 18–24 h of serum starvation in media containing 0.5% FBS, growth factors or 10% FBS were added. The concentrations of $VEGF_{165}$ added ranged from 5 pg/ml to 300 ng/ml; $PlGF_{152}$ concentrations were between 5.12 ng/ml and 3.2 μg/ml; the concentrations of VEGF-C were 40 ng/ml and 4 μg/ml. Following stimulation for 12–16 h at 37°C, [^{3}H]thymidine (1 mCi/ml; 5 Ci/mmol) was added for a final concentration of 1 μCi/ml and incubation proceeded at 37°C for 4 h. Removal of the media and several PBS washes were succeeded by trichloroacetic acid (TCA) precipitation. Following the removal of TCA, cells were then lysed with 0.2 M NaOH, 1% SDS, transferred to scintillation vials and neutralized with 2 M Na_2OAc, pH 4.0. The samples were counted using the tritium channel.

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