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SPECIFICITY OF VASCULAR ENDOTHELIAL CELL GROWTH FACTOR RECEPTOR LIGAND BINDING DOMAINS

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Vascular endothelial cell growth factor binds with high affinity to FLT and KDR, two homologous tyrosine kinase receptors expressed on vascular endothelial cells. Placental growth factor, a vascular endothelial cell growth factor homologue, also binds with high affinity to the extracellular domains of FLT but not to the extracellular region of KDR. Vascular endothelial cell growth factor binds competitively with placental growth factor to the extracellular ligand binding domains of FLT, indicating that both ligands probably complex to overlapping or identical regions of this receptor.

Placental growth factor (PlGF) is a secreted endothelial cell mitogen (1,2) that is 53% identical in amino acid sequence to vascular endothelial cell growth factor (VEGF) (3-5). PlGF and VEGF exist as glycosylated homodimers that exhibit homology, especially in their cysteine residue pattern, with PDGF A and B subunits. Two receptors that bind VEGF with high affinity, FLT (6) and KDR (7), have been identified. These molecules are type III tyrosine kinase receptors with extracellular domains that are composed of 7 immunoglobulin-like repeats. We recently described a naturally encoded soluble form of the FLT receptor (sFLT), generated by splice skipping, that binds VEGF with the same high affinity as the membrane spanning form of the receptor and inhibits VEGF induced mitogenesis of human umbilical vein endothelial cells (8).

Hauser and Weich (2) report that PlGF stimulates bovine aortic endothelial cells to divide and competes for VEGF binding to fetal bovine aortic endothelial cells. Here we show that PlGF binds with high affinity to the extracellular region of FLT but not KDR. VEGF competes efficiently for

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Abbreviations: PlGF, placental growth factor; VEGF, vascular endothelial cell growth factor, PAGE, polyacrylamide gel electrophoresis.

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PlGF binding to FLT whereas PlGF competes for VEGF binding but with lower efficiency.

MATERIALS AND METHODS

Reagents - Bis(sulfosuccinimidyl)suberate (BS^3) crosslinking reagent was from Pierce. Na[^{125}I] (14.7 mCi/ug) was obtained from Amersham.

Recombinant Proteins - Human sFLT was produced in Sf9 insect cells using the baculovirus expression system (Invitrogen) and purified as described (8). The extracellular region truncated between the 6th and 7th immunoglobulin-like domain at amino acid number 664 (lacking the transmembrane sequence and intracellular kinase domains) of human recombinant KDR (Ex-KDR), as well as $VEGF_{165}$ and PlGF-1/$PlGF_{131}$ (2) were produced using the baculovirus system and purified to homogeneity (Wang, G., DiSalvo, J., Kendall, R. L., Sullivan, K. A., Schaeffer, M-T. and Thomas, K. A., unpublished results).

Iodination - Pure recombinant human VEGF and PlGF were iodinated by the chloramine-T method (9) as described (8). Both growth factors were iodinated to specific activities between 3×10^5 - 9×10^5 cpm/ng. After iodination, VEGF and PlGF were stored at 4 °C in PBS containing 1 mg/ml gelatin.

Binding and Crosslinking - The 96 well plate binding assay for sFLT was done essentially as described (8). VEGF or PlGF binding to either sFLT or Ex-KDR as well as determination of the binding affinity of VEGF to Ex-KDR were evaluated by covalent crosslinking. Purified Ex-KDR and sFLT were each allowed to bind either [^{125}I]VEGF or [^{125}I]PlGF at 25 °C for 1 hr in a final volume of 25 µl in binding buffer (10 mM Hepes, pH 7.4, 0.01% BSA, 100 mM NaCl) with or without an excess of the appropriate unlabeled ligand. Competition binding was accomplished by incubation in the presence of various concentrations of unlabeled VEGF (0.1 - 400 nM). The reactions were then crosslinked with 1 mM BS^3 at 25 °C for 15 min followed by the addition of boiling Laemmli sample buffer (10). The crosslinked products were analyzed by SDS/7.5% PAGE and the complexes were visualized using a PhosphoImager (Molecular Dynamics, Sunnyvale, CA). In the competition crosslinking experiments the amount of radioactivity contained in the Ex-KDR/[^{125}I]VEGF complex as well as the uncomplexed [^{125}I]VEGF were quantified using the PhosphoImager.

RESULTS

VEGF binds to both sFLT and Ex-KDR whereas PlGF binds only to sFLT - To determine if sFLT and Ex-KDR bind VEGF and PlGF with high affinity, purified sFLT and Ex-KDR were each incubated with either [^{125}I]VEGF or [^{125}I]PlGF, covalently crosslinked and high molecular mass complexes were resolved by SDS/PAGE. sFLT forms high molecular mass complexes with both VEGF (Fig. 1A, lane 1) and PlGF (Fig. 1A, lane 4) whereas Ex-KDR forms complexes with VEGF (Fig. 1B, lane 1) but not with PlGF (Fig. 1B, lane 3). The positions of the monomer (one VEGF dimer bound to one receptor molecule)

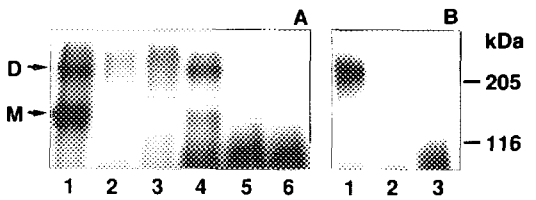

Fig. 1. Crosslinking analysis of VEGF and PlGF binding to sFLT and Ex-KDR. **A)** sFLT (0.5 ng) was incubated with 0.3 ng of either [^{125}I]VEGF (lanes 1-3) or [^{125}I]PlGF (lanes 4-6) plus a 100-fold excess of unlabeled VEGF (lanes 2 and 6) or PlGF (lanes 3 and 5). **B)** Ex-KDR (10 ng) was incubated with [^{125}I]VEGF (lanes 1 and 2) or [^{125}I]PlGF, (lane 3) in the presence of an excess of unlabeled VEGF (lane 2). Reactions were carried out in a total volume of 25 µl at 23 °C for 1 hr. Complexes were crosslinked for 15 min and analyzed by SDS/7.5% PAGE. The locations of standards in the mass range of the crosslinked products and of ligands crosslinked to either 2 (dimer, D) or 1 (monomer, M) sFLT molecules are marked.

and dimer (one VEGF dimer bound to two receptor molecules) are indicated. These radiolabeled complexes can be competed by an excess of the same unlabeled VEGF (Fig. 1A, lane 2; Fig. 1B, lane 2) or PlGF (Fig. 1A, lane 5) and thus are specific. PlGF is able to compete for VEGF binding to the sFLT receptor (Fig. 1A, lane 3) and VEGF can compete for PlGF binding to this receptor (Fig. 1A, lane 6). PlGF is not able to compete for [^{125}I]VEGF binding to Ex-KDR (data not shown).

The affinity of VEGF for Ex-KDR was determined by a crosslinking competition binding assay since the Ex-KDR receptor binds poorly to 96 well plates. A constant amount of [^{125}I]VEGF was bound to Ex-KDR in the presence of increasing concentrations of unlabeled VEGF (Fig. 2). The concentration of unlabeled VEGF required to displace 50% of the total [^{125}I]VEGF binding is approximately 1 nM, which is similar to the apparent K_d for the membrane form of KDR (7,11).

Competition between PlGF and VEGF for binding to sFLT - Competitive binding of VEGF and PlGF to sFLT was analyzed by the 96 well plate binding assay. A constant amount of either [^{125}I]VEGF or [^{125}I]PlGF was bound to immobilized sFLT in the presence of increasing amounts of either unlabeled VEGF or PlGF (Fig. 3). Binding of [^{125}I]VEGF is 50% displaced by 30 pM of unlabeled VEGF in close agreement with the previously reported K_d values of VEGF binding to sFLT and FLT (8,11). In comparison, 50% of the binding of [^{125}I]PlGF to sFLT is displaced by only 10 pM of VEGF. Approximately 110 pM of unlabeled PlGF displaces 50% of [^{125}I]PlGF binding to sFLT in agreement with saturation binding experiments (data not shown). However, an approximately 5-fold higher concentration of PlGF (~550 pM) is required to displace 50% of the [^{125}I]VEGF binding to sFLT. These data indicate that VEGF and PlGF probably compete for the same site on sFLT at which VEGF binds

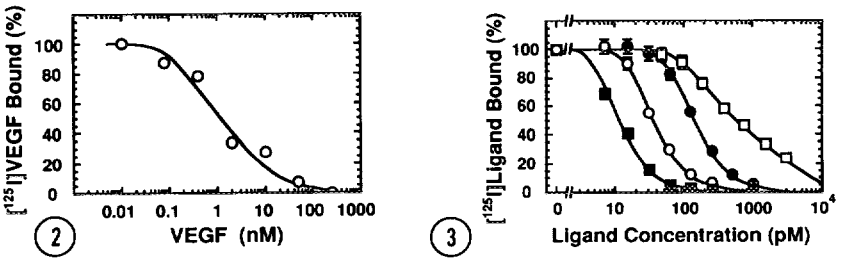

Fig. 2. Binding affinity of VEGF to Ex-KDR. [^{125}I]VEGF (0.5 ng) was mixed with purified Ex-KDR (10 ng) and incubated in the presence of increasing concentrations of unlabeled VEGF at 23 °C for 1 hr then crosslinked with BS^3 for 15 min in a total volume of 25 µl; reaction products were separated by SDS/7.5% PAGE and quantified using a PhosphoImager. Results shown are the means of three separate experiments.

Fig. 3. Cross competition of PlGF and VEGF binding to sFLT. A constant amount of [^{125}I]VEGF (50 pg/100 µl) was bound for 3 hr at 23 °C to ~200 ng of immobilized sFLT in the presence of increasing concentrations of either unlabeled VEGF (O) or PlGF (□) or a constant amount of [^{125}I]PlGF (50 pg/100 µl) was bound in the presence of increasing concentrations of unlabeled VEGF (■) or PlGF (●). Zero competitor concentration corresponds to 100% binding and all data points are the averages of triplicate determinations ± SD.

with ~4-fold higher affinity than PlGF. Crosslinking competition experiments with sFLT give similar results (data not shown).

DISCUSSION

Hauser and Weich (2) reported that PlGF is mitogenic for bovine aortic endothelial cells and can compete with 50% of VEGF binding to fetal bovine aortic endothelial cells. One interpretation of these data is that PlGF can only compete for VEGF binding to one of the two known VEGF receptors. However, relative binding affinities and specificity were not evaluated in these studies.

Here we show that VEGF binds to the extracellular domains of both FLT and KDR with high affinity. VEGF binding to the extracellular portion of these receptors has similar binding affinities to those reported for VEGF binding to the membrane bound form of these receptors (7,11). PlGF, however, only binds to the extracellular domain of FLT with high affinity and does not bind to the equivalent extracellular region of KDR. VEGF is able to compete efficiently for PlGF binding to sFLT whereas PlGF competes less efficiently for VEGF binding. These binding data demonstrate that VEGF complexes with sFLT somewhat tighter than does PlGF. Competitive binding infers that the VEGF and PlGF sites on sFLT are probably either overlapping or identical.

The results presented here support the hypothesis that the VEGF/PlGF ligand - FLT/KDR receptor system might in some ways be functionally similar to the weakly homologous PDGF system. PDGF BB homodimers bind to both PDGF α and β receptor subunits whereas PDGF AA homodimers can only bind to PDGF α receptor subunits. In an analogous fashion, VEGF binds to the extracellular domains of both the FLT and KDR receptors whereas PlGF only binds to the FLT ligand binding region. Therefore, the binding specificities of VEGF and PlGF are superficially similar to those of PDGF BB and AA, respectively. In addition, the dual and single ligand selectivities of FLT and KDR resemble those of the PDGF α and β subunits, respectively. In the PDGF system the formation of receptor and ligand heterodimers have been demonstrated (12) which might enable more extensive modulation of ligand stimuli and receptor responses. However, because of the distant homology between VEGF/PlGF and PDGF systems, the existence of VEGF/PlGF ligand and FLT/KDR receptor heterodimers is possible but not obvious.

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