RESEARCH COMMUNICATION

Synthesis and physiological activity of heterodimers comprising different splice forms of vascular endothelial growth factor and placenta growth factor

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Vascular endothilial growth factor (VEGF) and placenta growth factor (PlGF) are members of a dimeric-growth-factor family with angiogenic properties. VEGF is a highly potent and specific mitogen for endothelial cells, playing a vital role in angiogenesis *in vivo*. The role of PlGF is less clear. We expressed the monomeric splice forms VEGF-165, VEGF-121, PlGF-1 and PlGF-2 as unfused genes in *Escherichia coli* using the pCYTEXP expression system. *In vitro* dimerization experiments revealed that both homo- and hetero-dimers can be formed from these monomeric proteins. The dimers were tested for their ability to promote capillary growth *in vivo* and stimulate DNA synthesis in cultured human vascular endothelial cells. Heterodimers comprising different VEGF splice forms, or combinations of VEGF/PlGF splice forms, showed mitogenic activity. The results demonstrate that four different heterodimeric growth factors are likely to have as yet uncharacterized functions *in vivo*.

INTRODUCTION

Angiogenesis, the growth of new blood vessels, plays an important role in many physiological processes, including embryogenesis, wound healing and tissue or organ regeneration [1]. During the course of angiogenesis, quiescent endothelial cells begin with the localized degradation of their basement membrane and form new capillary blood vessels by sprouting into the surrounding stroma. The overall process is controlled by several factors which are able to stimulate vascular-endothelial-cell proliferation and migration [2]. Of these, vascular endothelial growth factor (VEGF) shows by far the highest specificity for vascular endothelial cells [3–5]. In addition to its angiogenic properties, VEGF can also induce vascular permeability, and is accordingly also known as vascular permeability factor. More recently, a gene encoding a VEGF-related factor was cloned from a placental cDNA bank [6]. This new protein, called placenta growth factor (PlGF), is synthesized in only a limited number of cell types and tissues, including human umbilical-vein endothelial cells and placenta.

VEGF and PlGF have been described as glycosylated, disulphide-linked homodimers that share 40% amino acid sequence identity [6,7]. They are also 20% identical with platelet-derived growth factor (PDGF) A and B peptides, whereby eight cysteine residues are strictly conserved. As in the case of PDGF, alternative splicing generates multiple forms of VEGF and PlGF. Human VEGF exists in four different mature forms, comprising 206,189,165, and 121 amino acids respectively [8]. VEGF-206 and VEGF-189 each contain a highly basic 24-amino-acid insert that promotes tight binding to heparin and is therefore thought to mediate interactions with heparan proteoglycans on cellular surfaces and within extracellular matrices. Indeed, secretion of the two larger isoforms leaves them primarily associated with the cell surface. Differential splicing of human PlGF mRNA leads to the production of two isoforms, PlGF-1 (132 amino acids) and PlGF-2 (153 amino acids). The additional 21 amino acids of the longer form constitute a basic region that may perform the same function as the basic insert of VEGF-165 [9]. It remains unclear whether the alternative splice forms of VEGF and PlGF have different biological functions. Two structurally similar receptor tyrosine kinases, namely kinase-insert-domain-containing receptor (KDR) and Fms-like tyrosine kinase (Flt-1), have been shown to bind VEGF [10,11]. The significance of each receptor subtype in terms of the functions of VEGF has yet to be determined. While the KDR receptor seems to mediate VEGF-induced mitogenic effects, the Flt-1 receptor may not in itself be able to perform this function [12,13]. PlGF, on the other hand, binds with high affinity to Flt-1, but not to the KDR receptor [14].

Here we describe the expression of VEGF and PlGF genes in *E. coli* as well as the purification and dimerization of the resulting proteins. This new approach allows us to generate sufficient quantities of the respective proteins for structural and functional analysis. Using the described *in vitro* techniques, we are able to show that not only homodimers, but also a range of heterodimers, can readily be formed by dimerization of the respective monomeric splice forms. This demonstration of the formation of biologically active VEGF and PlGF heterodimers indicates that there is a family of heterodimeric growth factors with as-yet-unidentified functions.

EXPERIMENTAL

Plasmid construction and gene expression

Standard procedures for the preparation, cloning and sequencing of DNA fragments were used as described in [15]. The cDNA

Abbreviations used: Flt 1, Fms-like tyrosine kinase; KDR, kinase insert domain-containing receptor; MVE cells, microvascular endothelial cells; PlGF, placenta growth factor; VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; CAM, chorioallantoic membrane; FCS, foetal-calf serum.

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sequences encoding VEGF [16] and PlGF [9] were inserted, as *Eco*RI/*Sal*I or *Eco*RI/*Bam*HI fragments respectively, into pCYTEXP1 [17]. Using the resulting plasmids, an *Nde*I site, and thus a start codon, was introduced into the respective sequences at sites corresponding to the N-termini of the mature VEGF and PlGF reading frames by means of *in vitro* mutagenesis. The single-stranded DNA for this procedure was prepared from pCYTEXP1 derivatives directly using the helper phage M13K07 [17,18]. The *Nde*I/*Xba*I fragments of these plasmids, containing the genes encoding the mature VEGF and PlGF chains, were finally introduced into pCYTEXP3 [19]. The *E. coli* strain JM 109 [20] was used as the host for expression experiments. Cells containing derivatives of pCYTEXP1 or pCYTEXP3 were grown on TB medium (12 g/l bacto-tryptone, 24 g/l bacto-yeast extract, 4 ml/l glycerine, 17 mM KH_2PO_4 and 72 mM K_2HPO_4) in the presence of 100 ng/ml ampicillin at 30 °C. A temperature shift to 42 °C was used to induce expression of the VEGF and PlGF genes once the transformed cultures had reached an A_{550} of 0.6–0.8.

Purification and dimerization of VEGF and PlGF monomers

After 4 h of growth of transformed cultures at 42 °C, inclusion bodies were isolated [21], and solubilized in 6 M guanidinium chloride. The thiol groups were protected by S-sulphonation [22]. Samples (5 ml each) were separated by gel filtration on a Superdex 75HR columm (26/60; Pharmacia). VEGF- and PlGF-containing fractions were dialysed for 16 h at 4 °C against 50 mM Tris/HCl, pH 8.0. The concentration of purified protein was determined by the BCA (bicinchoninic acid) method [23]. After dialysis the monomeric proteins were allowed to dimerize over a period of 24 h in the presence of GSH/GSSG (molar ratio 5:1) in a solution of urea. The conditions for dimerization were similar to those described previously [19,22], except that the urea concentrations were adjusted as follows: VEGF homodimers, 2 M; VEGF/PlGF heterodimers, 3 M; PlGF homodimers, 4 M. Dimer preparations were extensively dialysed against 25 mM Tris/Mes, pH 5.0, and loaded on to a Mono-S (Pharmacia) cation-exchange column. After washing the column with 25 mM Tris/Mes, pH 5.0, the bound dimers were eluted using a linear gradient of NaCl. Depending on the type of growth factor, VEGF and PlGF homo- and hetero-dimers were eluted between 0.3 and 0.6 M NaCl. Protein-containing fractions were analysed by means of SDS/16 %-PAGE under reducing and non-reducing conditions. Western blotting was performed once the proteins had been transferred to a nylon membrane using a semi-dry electroblotter. The membrane was blocked with 2 % BSA/TBS-T (20 mM Tris/HCl, pH 8.0, 150 mM NaCl and 0.01 % Tween-20) and incubated with anti-VEGF and/or anti-PlGF rabbit polyclonal antibodies, followed by anti-rabbit IgG polyclonal antibodies labelled with alkaline phosphatase and revealed by using 5-bromo-4-chloroindol-3-yl phosphate (*p*-toluidine salt) and Nitroblue Tetrazolium chloride. Antibodies were prepared according to standard techniques [24] using monomeric VEGF and PlGF proteins derived from the *E. coli* strains described.

In vitro and *in vivo* mitogenic activities

The *in vitro* mitogenic activities of VEGF and PlGF homo- and hetero-dimers were assayed as a function of concentration using human microvascular endothelial (MVE) cells (Clonetics). MVE were introduced at 2×10^4 cells·ml^{-1} on to gelatinized 24-well plates in endothelial growth medium-microvascular containing 5 % foetal-calf serum (FCS) and allowed to grow for 18 h. The cells were then shifted to starvation conditions by resuspending them in basal medium (EBM) supplemented with 2 % FCS. At 24 h after this shift, different amounts of the dimerized growth factors were added to the quiescent cells. After a further 18 h, [^{3}H]thymidine (Amersham International) was added to a final concentration of 1 μCi·ml^{-1}, followed by incorporation over a period of 6 h. Incorporated radioactivity was finally measured using a liquid-scintillation counter subsequent to harvesting and washing of the cells. The *in vivo* effects of the growth factors were studied using the chorioallantoic membrane (CAM) of 13-day-old chicken embryos. Fertilized eggs of the White Leghorn chick were incubated at 37.8 °C and 80 % humidity. On day 5, a window was cut in the shell and the embryos checked for normal development. The window was sealed with Sellotape and the eggs were further incubated until day 13 of development. The freeze-dried growth-factor pellets were dissolved in sterile water and 2–4 μg/5 μl were placed on a tissue-culture coverslip (Nunc, Naperville, IL, U.S.A.) covering an area of about 5 mm^2. After air-drying, the inverted coverslips were placed on the CAM of the 13-day-old chick embryos. After a further 3 days of incubation, the CAMs were fixed in 2.0 % formaldehyde and 3.0 % glutaraldehyde in 0.12 M cacodylate buffer. They were photographed using a stereomicroscope (Wild M8). Controls were performed using carrier discs which had not been treated with growth-factor solutions [25].

RESULTS AND DISCUSSION

Preparation and purification of VEGF and PlGF homo- and hetero-dimers

Expression of the VEGF and PlGF genes in *E. coli* was achieved using the vector pCYTEXP3 [19]. We used continuous reading frames which were equivalent to the known mature mRNA species that are generated via splicing of the VEGF and PlGF pre-mRNAs in higher eukaryotic cells. The recombinant expression constructs of the splice forms VEGF-121, VEGF-165, PlGF-1 and PlGF-2 (Figure 1) all directed the synthesis of large amounts of the respective proteins, which accumulated as inclusion bodies. The monomeric growth-factor proteins were isolated from the inclusion-body preparations by means of a one-

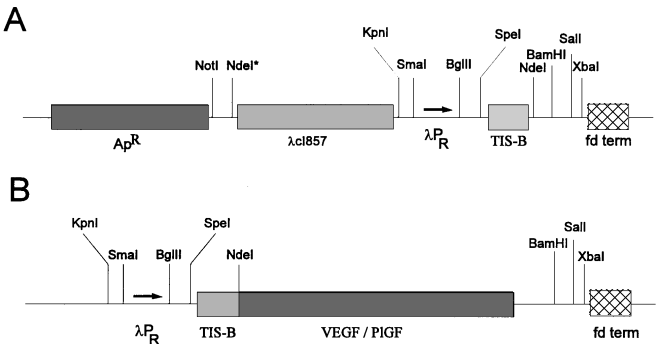

Figure 1 Plasmid maps of the growth-factor expression constructs

(**A**) The expression vector used was pCYTEXP3 [19]. The expression cassettes comprised the bacteriophage λ P_R promotor, a synthetic 5′ non-coding initiation sequence (TIS-B), the phage fd transcriptional terminator and the bacteriophage λ cI857 repressor gene. (**B**) Monocistronic TIS-B/VEGF or TIS-B/PlGF expression vectors. In order to obtain direct cytoplasmic synthesis of mature human VEGF and PlGF in *E. coli*, the genes were inserted via an *Nde*I restriction site that had been introduced at the start codon of each reading frame.

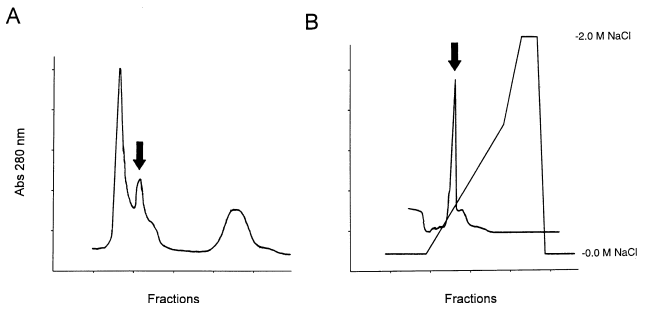

Figure 2 Purification of monomeric and dimeric growth factors by means of FPLC

Solubilized inclusion bodies were loaded on to a Superdex 75HR (26/60 Pharmacia) column, which was run with 4 M guanidinium chloride/50 mM Tris/HCl, pH 7.6 (**A**). The dimeric forms were purified using a Mono-S (Pharmacia) ion-exchange column run with 25 mM Tris/Mes, pH 5.0 (**B**). Elution was achieved using a 0–2 M NaCl gradient. The monomer and dimer peaks are indicated by arrows. Abbreviation: Abs, absorbance at.

step purification procedure (Figure 2A). The exact dimerization conditions had to be optimized for each combination of monomeric growth factors (see the Experimental section). The resulting dimers of the growth factors were purified from monomeric proteins and small amounts of other protein contaminants by means of ion-exchange chromatography (Figure 2B). The elution profile from the ion-exchange column showed a single peak equivalent to the dimeric material, while monomeric growth factors and other proteins were found mainly in the column flow-through fractions. The elution conditions were adjusted according to the properties of the respective dimers (see the Experimental section).

Using the above procedure, large amounts of homodimers of the growth factors VEGF and PlGF can readily be prepared in a highly pure state (Figure 3). Moreover, we could also show that heterodimers could be formed between different monomers. Thus the monomers described in the present work were found to dimerize to form the heterodimers VEGF-121/165, VEGF-121/PlGF-1, VEGF-165/PlGF-1, and VEGF-165/PlGF-2. These heterodimers could be separated from monomers and homodimers by means of ion-exchange chromatography . The VEGF/PlGF heterodimers showed distinct mobilities in SDS/PAGE gels under non-reducing conditions compared with the homodimers. Western-blot analysis, using VEGF- and PlGF-specific polyclonal antibodies, confirmed the presence of both VEGF and PlGF monomers in each of the heterodimers (Figure 3). Moreover, reduction of the dimeric growth factors releases the component monomers, whose identities can also be readily determined on the basis of electrophoretic mobilities and reaction with the appropriate antibodies (Figure 3).

In vitro and *in vivo* activity of VEGF and PlGF homo- and heterodimers

We studied the ability of growth factors to stimulate DNA synthesis in MVE cells *in vitro* (Figure 4). The exclusively VEGF-containing homo- and hetero-dimers were the most potent mitogens for these cells, yielding an ED_{50} of 3–5 ng/ml. These VEGF dimers promoted increasing levels of thymidine incorporation over the whole concentration range from 2 to 10 ng/ml. The VEGF/PlGF heterodimers showed similar activities to those of the VEGF homodimers, giving an ED_{50} of 6–10 ng/ml. The homodimeric PlGF-1 and PlGF-2 preparations showed no detectable activity in this assay system.

The dimeric growth factors were also tested in an assay designed to assess their ability to promote blood capillary growth.

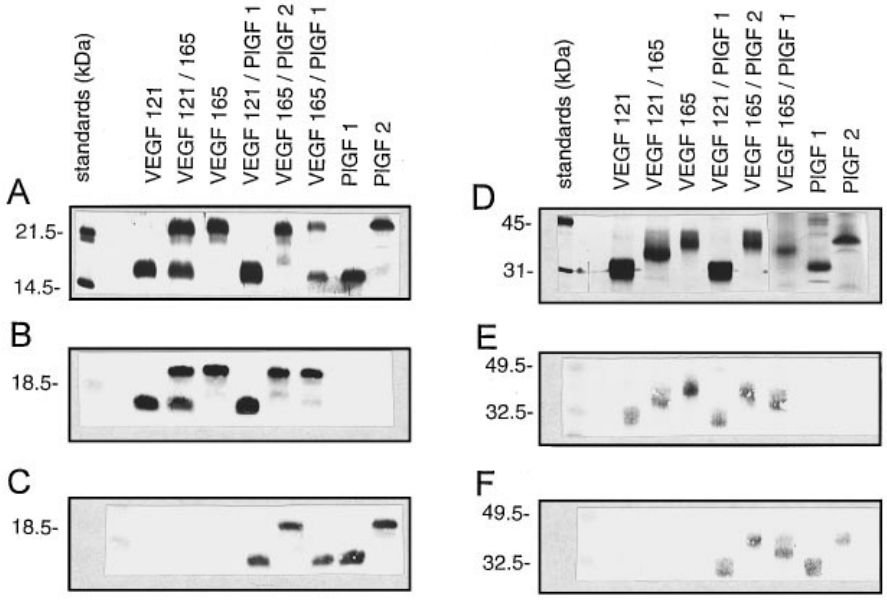

Figure 3 Western-blot analysis of purified VEGF, VEGF/PlGF and PlGF dimers

Purified growth-factor dimers derived from the monomeric polypeptides VEGF-121, VEGF-165, PlGF-1 and PlGF-2 were subjected to electrophoresis through an SDS/16%-polyacrylamide gel under reducing (**A**) and non-reducing (**D**) conditions, followed by silver staining. Protein standards and samples were loaded as indicated. The dimeric proteins tended to run as relatively diffuse bands. VEGF, VEGF/PlGF, and PlGF dimers were analysed using specific antisera after electrophoresis through SDS/polyacrylamide gels (loaded as in **A** and **D** respectively) under reducing (**B** and **C**) and non-reducing (**E** and **F**) conditions followed by electrophoretic transfer on to nylon membranes. The membranes were incubated with rabbit anti-VEGF (**B** and **E**) or anti-PlGF(**C** and **F**) polyclonal antibodies and alkaline phosphatase-labelled goat anti-rabbit IgG antibody.

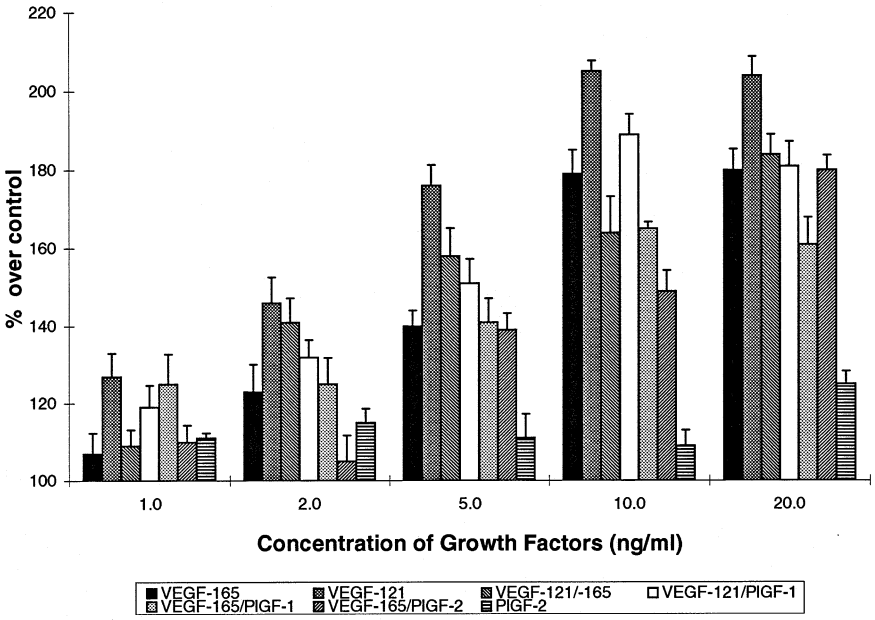

Figure 4 Endothelial-cell proliferation assay

The vascular endothelial cells were stimulated by the addition of purified growth factors. VEGF, VEGF/PlGF or PlGF dimers were added to the cells giving the indicated final concentrations, and measurements of methyl[^{3}H]thymidine incorporation were performed. Each value represents the mean of measurements obtained with triplicate cultures in a representative experiment. DNA synthesis in the control culture was set to 100%.

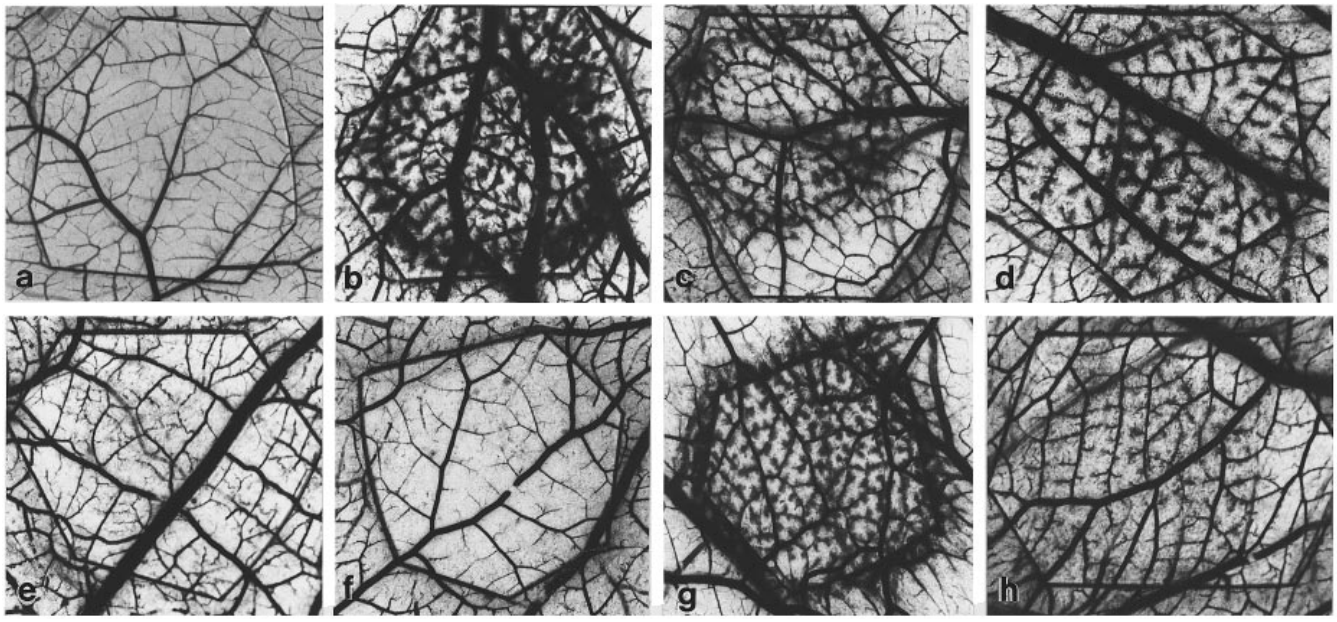

Figure 5 Effects of VEGF and PlGF homo- and hetero-dimers applied to the *CAM* of 13-day-old chicken embryos

(**a**) Control; the carrier disc does not induce alterations in the vascular tree. (**b**) VEGF-121; brush-like capillaries formed beneath the carrier disc. (**c**) VEGF-165; brush-like capillaries. (**d**) VEGF 121/165 heterodimers; formation of brush-like capillaries. (**e**) PlGF-1; there is no increase in vascular density beneath the carrier disc. (**f**) PlGF-2; the vascular pattern is not detectably affected. (**g**) VEGF 121/PlGF-1 heterodimer; formation of brush-like vessels. (**h**) VEGF-165/PlGF-2 heterodimer; formation of brush-like vessels. Magnification $\times 6$.

At 3 days after the application of the growth factors to the chicken CAM, the angiogenic response was evaluated using a stereomicroscope (Figure 5). The carrier disc alone had no effect on vascular structure. Homodimeric VEGF-121 and VEGF-165 induced the development of brush-like vessels in the precapillary region. The same effect was observed after application of the VEGF-121/165 heterodimer. In contrast, neither PlGF-1 nor PlGF-2 were found to be angiogenic in this system. There was no

formation of brush-like vessels in response to PlGF, although the capillaries in the specimens treated with PlGF homodimers appeared to be slightly dilated in comparison with the control. The VEGF-121/PlGF-1 and VEGF-165/PlGF-2 heterodimers, on the other hand, were clearly angiogenic, whereby the response was somewhat weaker than that observed with the exclusively VEGF-containing dimers. Further experiments revealed that VEGF-165/PlGF-1 has similar angiogenic properties to those of the other heterodimers (results not shown).

Functional significance of heterodimeric growth factors

In the present study we have addressed an as yet hardly explored area of growth-factor structure and function: can monomers of the VEGF/PlGF group form heterodimers, and are such products biologically active? An important prerequisite for investigations of this issue is the availability of appropriate quantities of the respective monomeric splice forms. The *E. coli* expression system we describe provides a solution to this problem. Moreover, we have been able to develop an effective strategy for the preparation of heterodimers. Our *in vitro* studies demonstrate that a range of heterodimers can be formed, both within the VEGF group and between the VEGF and PlGF monomer types. Finally, we have been able to show that heterodimeric growth factors comprising VEGF and/or PlGF splice forms are biologically active.

The observation that VEGF and VEGF/PlGF heterodimers are readily formed *in vitro* raises the question of their significance *in vivo*. At least in terms of structural versatility, this constitutes a situation analogous to the formation of three different forms of dimeric PDGF [19,22,26]. In the latter case, all three isomeric forms can be isolated from human platelets, whereby the AB form is the most abundant species [27]. The significance of the formation of such heterodimers is likely to be related to the fact that there is more than one type of receptor population. The PDGF α-receptor binds all three PDGF isoforms with high affinity, whereas the β-receptor shows high-affinity binding to only the BB form [28,29]. Ligand binding induces receptor dimerization, which in the case of PDGF-AB involves formation of an α/β dimer. Analogously, receptor heterodimers (KDR/Flt-1) may also be induced to form by cellular interactions with VEGF/PlGF heterodimers. The formation of such receptor heterodimers could, in turn, initiate an alternative response to that triggered by the binding of homodimeric growth factors.

Fully consistent with our findings, DiSalvo and colleagues [30] have identified heterodimeric VEGF/PlGF in a rat glioma cell line, while Cao and colleagues found the VEGF-165/PlGF-1 heterodimer in the conditioned media of human tumour-cell lines [31]. Our work has shown that at least four different heterodimers can be formed *in vitro*, raising the possibility that all of them are functionally significant *in vivo*. With the help of the strategy described here, it should now be possible to achieve rapid progress towards defining the physiological roles of these newly identified growth-factor forms.

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