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Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor

(growth factors/cancer/angiogenesis/protein processing)

DOMENICO MAGLIONE*, VALENTE GUERRIERO*, GIUSEPPE VIGLIETTO*, PASQUALE DELLI-BOVI†, AND M. GRAZIELLA PERSICO*‡

*International Institute of Genetics and Biophysics, Consiglio Nazionale della Ricerce, Via Marconi 10, 80125 Naples, Italy; and †Dipartimento di Genetica, Biologia Generale e Molecolare, Università di Napoli, Via Mezzocannone 8, 80100 Naples, Italy

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ABSTRACT **A human cDNA coding for a protein related to the vascular permeability factor (VPF) was isolated from a term placenta cDNA library; we therefore named its product placenta growth factor (PlGF). PlGF is a 149-amino-acid-long protein and is highly homologous (53% identity) to the platelet-derived growth factor-like region of human VPF. Computer analyses reveal a putative signal peptide and two probable N-glycosylation sites in the PlGF protein, one of which is also conserved in human VPF. By using N-glycosidase F, tunicamycin, and specific antibodies produced in both chicken and rabbit, we demonstrate that PlGF, derived from transfected COS-1 cells, is actually N-glycosylated and secreted into the medium. In addition, PlGF, like VPF, proves to be a dimeric protein. Finally, a conditioned medium from COS-1 cells containing PlGF is capable of stimulating specifically the growth of CPA, a line of endothelial cells, *in vitro*.**

Formation of new blood vessels, angiogenesis, is involved in both normal and pathological events such as embryogenesis, inflammation, wound healing, neoplasia, progression of ocular diseases, and rheumatoid arthritis. In particular, angiogenesis is an important process for the growth of tumors (1, 2) as recently demonstrated in rat primary tumors induced by chemical agents (3). Furthermore, angiogenesis may favor the formation of cancer metastasis (4, 5).

Angiogenesis is a complex process involving several steps, including migration and proliferation of endothelial cells (1, 6). These steps are stimulated by a variety of angiogenic growth factors (7, 8), such as the platelet-derived endothelial cell growth factor (9), the transforming growth factors α (10) and β (11), and the vascular permeability factor (VPF) (12, 13) also called vascular endothelial growth factor (VEGF) (14, 15). These growth factors appear to fall into two groups: (*i*) those acting directly on endothelial cells and (*ii*) those acting indirectly by inducing host cells to release specific endothelial growth factors (7, 16). One member of the first group is VPF/VEGF, a dimeric protein isolated from conditioned medium of different cell types. Besides angiogenic activity, VPF/VEGF displays the physiological function of increasing the permeability of capillary vessels to different macromolecules (13, 17).

In this paper, we report the sequence of a human cDNA, isolated from a term placenta cDNA library. This cDNA codes for a VPF-related protein of 149 amino acids, which we termed placenta growth factor (PlGF).§ The protein has a very strong similarity (53% identity) to the platelet-derived growth factor (PDGF)-like region of VPF/VEGF. Furthermore, different experimental approaches reveal that PlGF, like VPF/VEGF, is a secreted, N-glycosylated, and dimeric protein, capable of stimulating the growth of CPA endothelial cells *in vitro*.

MATERIALS AND METHODS

Screening and Sequencing of cDNA Clones. Clones of a human term placenta cDNA library (10^5) and of a choriocarcinoma cell line (JEG-3) (2×10^5) cDNA library constructed in λgt11, both made from poly(A)$^+$-enriched cytoplasmic RNA were screened by standard procedures (18). After small-scale phage DNA preparations, the two *Eco*RI fragments of the longest clone were separately subcloned in a pGEM1 vector (Promega) and sequenced in both directions by a modified dideoxynucleotide method (19).

Plasmid Construction. To obtain a recombinant clone containing the complete cDNA, the two *Eco*RI fragments of the phage insert were ligated together with the pGEM1 vector. The clones corresponding to the complete cDNA were detected by colony hybridization, using as probe an oligonucleotide (5′-GACCCTCAGGAATTCAGTGCCTTC-3′) overlapping the internal *Eco*RI site of the PlGF cDNA, as deduced by genomic DNA sequencing. A pSVL-PlGF plasmid was constructed by cloning a filled-in *Ava* I/*Bam*HI fragment of the PlGF DNA (from nucleotides 304–1182) in the *Sma* I site of the pSVL vector (Pharmacia). Thus, the PlGF transcription is under the simian virus 40 late promoter control.

To synthesize the PlGF protein in bacteria, we constructed a prokaryotic expression plasmid named pET-PlGF1, obtained by cloning in a pET-3 vector (Novagen, Madison, WI) a PlGF cDNA synthesized by the PCR technique. As template, we used the complete PlGF cDNA, and, as primers, we used two oligonucleotides. The first (5′-TCCTCCAAGGGGATCCTGGGTTAC-3′) was complementary to the PlGF cDNA sequence from nucleotides 787–768 except for an artificial *Bam*HI site (underlined), while the second (5′-CCTTGTCTGCTCATATGGGGAACG-3′) was identical to the sequence from nucleotides 404–421 of the PlGF cDNA except for an artificial *Nde* I site (underlined). After the PCR, the synthesized fragment was digested with *Nde* I and *Bam*HI restriction enzymes and then cloned in the corresponding single sites of the pET-3 vector.

Cell Cultures. All the cell lines were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) in a humidified atmosphere of 5% CO_2/95% air. COS-1 cells (10^6) (20) were transfected by the calcium phosphate method (21) using 5 μg of the pSVL-PlGF (see above) or pSVL vector (Pharmacia). Forty-eight hours after transfection, the cells were rinsed

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Abbreviations: PDGF, platelet-derived growth factor; PlGF, placenta growth factor; VEGF, vascular endothelial cell growth factor; VPF, vascular permeability factor; FCS, fetal calf serum.

‡To whom reprint requests should be addressed.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. X54936).

twice with phosphate-buffered saline (PBS; 137 mM NaCl/27 mM KCl/8 mM Na_2HPO_4/1.75 mM KH_2PO_4) and incubated with DMEM without FCS for an additional 24 hr. This conditioned medium was used as a source of unlabeled PlGF. To obtain labeled PlGF, 48 hr after transfection the cells were rinsed as described above and starved by incubation for 2 hr with Eagle's minimal essential medium (EMEM) without methionine. Subsequently, the medium was replaced by fresh EMEM containing 60 μCi (800 μCi/mmol; 1 Ci = 37 GBq) of [^{35}S]methionine per ml (DuPont/NEN). In some experiments, tunicamycin (100 μg/ml) (Sigma) was added in both the starvation and labeling phases. Four hours later, the radioactive medium was removed and stored after addition of phenylmethylsulfonyl fluoride to a final concentration of 1 mM. The cells were rinsed twice with PBS and treated with a Nonidet P-40 lysis buffer (50 mM Tris·HCl, pH 8/1% Nonidet P-40/300 mM NaCl) at 4°C for 1 hr. The cell lysates were collected and spun at 12,000 × g for 10 min at 4°C, and the supernatants were stored at −80°C until used. The promoting growth activity of PlGF was tested on different cell lines as described (22).

Production of Bacterial PlGF and Anti-PlGF Antibodies. The procedures used for bacterial culture and cell lysate preparation were as described (23). pET-PlGF1 or pET-3 plasmids were used to transform a JM109(DE3) bacterial strain (Promega). After loading the lysate onto a preparative SDS/15% polyacrylamide gel, the corresponding PlGF band was cut, electroeluted by "Elutrap" (Schleicher & Schuell), and precipitated with 2 vol of acetone for 30 min at −20°C. The precipitate was collected by spinning, vacuum-dried, and resuspended in PBS. A total of 70 μg of bacterial PlGF was used to immunize two chickens as described (24). The chicken antibodies were extracted and purified from the yolk by the polyethylene glycol (PEG) precipitation method (24). Two rabbits were immunized with a total of 300 μg of antigen resuspended in Freund's complete adjuvant by three subcutaneous injections.

Immunoassays. Immunoprecipitation assays were performed with 200 μl of labeled cell lysates or conditioned medium from transfected COS-1 cells and 10 or 15 μl of polyclonal anti-PlGF antibodies from rabbit or chicken, respectively, for 2 hr at room temperature and in some cases overnight at 4°C. The immunoreactions performed with antibodies from chicken eggs were further treated with 15 μl of rabbit anti-chicken IgG whole molecule (Sigma catalog no. C6778) for 1 hr at room temperature. The immunocomplexes were collected by protein A-Sepharose 4B beads (Pharmacia) and rinsed twice with 1.2 ml of a washing solution (PBS/0.01% Nonidet P-40/400 mM NaCl). The recovered immunoprecipitates were resuspended in 40 μl of SDS sample buffer (25), boiled for 8 min, and centrifuged for 5 min; the supernatant was loaded onto an SDS/12.5% polyacrylamide gel. In some experiments, the SDS sample buffer did not contain 2-mercaptoethanol. Immunoblotting was carried out by precipitating 2 ml of COS-1 transfected conditioned medium with 2 vol of acetone for 30 min at −20°C. The pellets were resuspended in 20 μl of SDS sample buffer with or without 2-mercaptoethanol and loaded onto an SDS/12.5% polyacrylamide gel followed by Western blotting. The nitrocellulose membrane was blocked with 3% (wt/vol) bovine serum albumin in PBS and incubated with anti-PlGF antibodies from chicken eggs at a dilution of 1:40 overnight at room temperature. Subsequently the filter was rinsed three times with washing solution and reblocked for 30 min; then it was incubated with a rabbit anti-chicken IgG peroxidase conjugated (Sigma) at a dilution of 1:1000 for 2 hr at room temperature. After three more rinses, staining was done with diaminobenzidine (0.75 mg/ml) (Sigma) in 0.1 M Tris·HCl, pH 7.6/0.03% H_2O_2/1.4 mM $CoCl_2$. The immunofluorescence staining of COS-1 transfected cells was carried out as described (22).

N-Glycosidase F Digestions. These reactions were performed with 200 μl of lysate or medium from COS-1 transfected cells and N-glycosidase F (glycopeptide N-glycosidase; glycopeptide glycanohydrolase, EC 3.2.2.18) (Boehringer Mannheim) according to the manufacturer's protocol.

RESULTS

Isolation and Characterization of cDNA. Previous screening of a human placenta cDNA library to isolate full-length glucose-6-phosphate dehydrogenase cDNA led to the identification of 16 different clones (26). Restriction map and sequencing analysis showed that one of these cDNA clones contained a 190-base-pair (bp) fragment at one end (sub32) with no apparent relationship with the glucose-6-phosphate dehydrogenase locus. This cDNA fragment is the starting point of this investigation.

The sub32 cDNA fragment was used as a probe to screen a cDNA library made with RNA extracted from choriocarcinoma cells. The longest insert present among the isolated clones was chosen to screen a term placenta cDNA library. Fig. 1 shows the nucleotide sequence of the insert present in one such clone. The insert, 1645 bp long probably corresponding to a full-length cDNA, presents an open reading frame of 447 bp potentially coding for a protein of 149 amino acids. The sequence surrounding the AUG codon at positions 322–324 is in agreement with the consensus sequence for the initiator AUG proposed by Kozak (27).

The 5′ untranslated region present in this clone is 321 nucleotides long and has a high G+C content (≈73%). The 3′ untranslated region, 877 bp long, has a lower G+C content (≈63%), and a similar G+C content is present in the coding region. Finally, a canonical polyadenylylation signal, AATAAA, is located at nucleotides 1620–1625.

PlGF Is a Secreted Protein Very Similar to the PDGF-Like Region of VPF. Comparison of the predicted 149-amino acid protein with a protein sequence data base (Microgenie; Beckman) revealed a strong similarity (53% identity) between amino acids from positions 39–132 of PlGF and amino acids from positions 38–131 of human VPF (a region containing the PDGF-like domain of VPF/VEGF) (13, 15) (Fig. 2). This similarity increases to ≈71% if the conserved amino acid substitutions are also considered. Because of this similarity, we believe that the cDNA we isolated codes for a putative growth factor synthesized in the placenta, so we termed our clone PlGF.

The hydrophobicity plot constructed according to the procedure of Kyte and Doolittle (28) (data not shown) reveals that PlGF, like VPF (13), presents a hydrophobic sequence at the N-terminal region, which may represent a signal peptide. This result is further confirmed by using the algorithm of Von Heijne (29), which predicts four probable cleavage sites of the signal peptide. One of them occurs between amino acids 20 and 21 of the PlGF leader region and shows the best score (data not shown). To confirm these theoretical data, we performed a series of immunoprecipitation experiments aimed at characterizing the PlGF protein.

As a source of PlGF protein, we used COS-1 cells transfected with the pSVL-PlGF plasmid. Fig. 3 shows the immunoprecipitation pattern obtained with transfected COS-1 cells and anti-PlGF antiserum produced in rabbits. The proteins of the COS-1 cells transfected with the pSVL-PlGF or pSVL plasmid were labeled with [^{35}S]methionine. A specific PlGF band of ≈25 kDa is evident (large arrow in Fig. 3) only in the COS(+) medium (lanes +) treated with immune anti-PlGF antiserum but not when the same medium was immunoprecipitated with preimmune rabbit serum (lanes P) or when the COS(−) medium (lanes −) was used. These results were further confirmed by using anti-PlGF antibodies extracted from chicken egg yolk (data not shown). These data indicate that PlGF is indeed a secreted protein.

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GGGATTCGGGCCGCCCAGCTACGGGAGGACCTGGAGTGGCACTGGGCGCCCGACGGACCATCCCCGGGACCCGCCTGCCCCTCGGCGCCCCGCCCCGCCGGGCCGCTCCC   110

CGTCGGGTTCCCCAGCCACAGCCTTACCTACGGGCTCCTGACTCCGCAAGGCTTCCAGAAGATGCTCGAACCACCGGCCGGGGCCTCGGGGCAGCAGTGAGGGAGGCGTC   220
                                                                                    AvaI
CAGCCCCCCACTCAGCTCTTCTCCTCCTGTGCCAGGGGCTCCCCGGGGGATGAGCATGGTGGTTTTCCCTCGGAGCCCCCTGGCTCGGGACGTCTGAGAAGATGCCGGTC   330
                                                                                      M  P  V

ATGAGGCTGTTCCCTTGCTTCCTGCAGCTCCTGGCCGGGCTGGCGCTGCCTGCTGTGCCCCCCCCAGCAGTGGGCCTTGTCTGCTGGGAACGGCTCGTCAGAGGTGGAAGT   440
 M  R  L  F  P  C  F  L  Q  L  L  A  G  L  A  L  P  A  V  P  P  Q  Q  W  A  L  S  A  G  N  G  S  S  E  V  E  V

GGTACCCTTCCAGGAAGTGTGGGGCCGCAGCTACTGCCGGGCGCTGGAGAGGCTGGTGGACGTCGTGTCCGAGTACCCCAGCGAGGTGGAGCACATGTTCAGCCCATCCT   550
  V  P  F  Q  E  V  W  G  R  S  Y  C  R  A  L  E  R  L  V  D  V  V  S  E  Y  P  S  E  V  E  H  M  F  S  P  S

GTGTCTCCCTGCTGCGCTGCACCGGCTGCTGCGGCGATGAGAATCTGCACTGTGTGCCGGTGGAGACGGCCAATGTCACCATGCAGCTCCTAAAGATCCGTTCTGGGGAC   660
C  V  S  L  L  R  C  T  G  C  C  G  D  E  N  L  H  C  V  P  V  E  T  A  N  V  T  M  Q  L  L  K  I  R  S  G  D

CGGCCCTCCTACGTGGAGCTGACGTTCTCTCAGCACGTTCGCTGCGAATGCCGGCCTCTGCGGGAGAAGATGAAGCCGGAAAGGTGCGGCGATGCTGTTCCCCGGAGGTA   770
 R  P  S  Y  V  E  L  T  F  S  Q  H  V  R  C  E  C  R  P  L  R  E  K  M  K  P  E  R  C  G  D  A  V  P  R  R

ACCCACCCCTTGGAGGAGAGAGACCCCGCACCCGGCTCGTGTATTTATTACCGTCACACTCTTCAGTGACTCCTGCTGGTACCTGCCCTCTATTTATTAGCCAACTGTTT   880
                                                                EcoRI
CCCTGCTGAATGCCTCGCTCCCTTCAAGACGAGGGGCAGGGAAGGACAGGACCCTCAGGAATTCAGTGCCTTCAACAACGTGAGAGAAAGAGAGAAGCCAGCCACAGACC   990

CCTGGGAGCTTCCGCTTTGAAAGAAGCAAGACACGTGGCCTCGTGAGGGGCAAGCTAGGCCCCAGAGGCCCTGGAGGTCTCCAGGGGCCTGCAGAAGGAAAGAAGGGGGC   1100
                                                                                  BamHI
CCTGCTACCTGTTCTTGGGCCTCAGGCTCTGCACAGACAAGCAGCCCTTGCTTTCGGAGCTCCTGTCCAAAGTAGGGATGCGGATCCTGCTGGGGCCGCCACGGCCTGGT   1210

GGTGGGAAGGCCGGCAGCGGGCGGAGGGGATCCAGCCACTTCCCCCTCTTCTTCTGAAGATCAGAACATTCAGCTCTGGAGAACAGTGGTTGCCTGGGGGCTTTTGCCAC   1320

TCCTTGTCCCCCGTGATCTCCCCTCACACTTTGCCATTTGCTTGTACTGGGACATTGTTCTTTCCGGCCGAGGTGCCACCACCCTGCCCCCACTAAGAGACACATACAGA   1430

GTGGGCCCCGGGCTGGAGAAAGAGCTGCCTGGATGAGAAACAGCTCAGCCAGTGGGGATGAGGTCACCAGGGGAGGAGCCTGTGCGTCCCAGCTGAAGGCAGTGGCAGGG   1540

GAGCAGGTTCCCCAAGGGCCCTGGCACCCCCACAAGCTGTCCCTGCAGGGCCATCTGACTGCCAAGCCAGATTCTCTTGAATAAAGTATTCTAGTGTGGAAACGC(A)n   1645
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FIG. 1. Nucleotide sequence of the human PlGF cDNA and predicted amino acid sequence of the PlGF precursor. Polyadenylylation site is indicated by double underlining; $(A)_n$ indicates the poly(A) tail. Restriction enzyme sites used to construct plasmid pSVL-PlGF are indicated and the putative N-glycosylation sites are underlined.

PlGF Is an N-Glycosylated Protein. The observed molecular mass of COS-derived PlGF (≈25 kDa), larger than that deduced from the PlGF amino acid sequence (16.7 kDa) or observed by *in vitro* translation (data not shown), indicates that PlGF is a processed protein. Amino acid sequence analyses reveal that two putative N-glycosylation sites (30) are present in the predicted protein (underlined in Figs. 1 and 2), one of which corresponds to the single site present in the human VPF (13). Thus, the molecular mass difference may be due to the addition of sugars. By conducting immunoprecipitation experiments using rabbit anti-PlGF antiserum and COS-1 transfected cell lysate, we observed that at least three additional PlGF-related bands may represent the above-mentioned molecular mass forms (small arrows in Fig. 3).

Moreover, we demonstrated by using tunicamycin, a specific inhibitor of N-glycosylation (31), and N-glycosidase F, an enzyme that cuts the bond between asparagine and

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  1  M P V M R L F P C F L Q L L A G L A L P A V P P Q Q W A L S  PlGF
     |       |                 | |   |   |             |   |
  1  M - - N F L L S W V H W S L A L L Y L H H A K W S Q A A P M  hVPF

 31  A - G N G S S E V E V V P F Q E V W G R S Y C R A L E R L V  PlGF
     |   |   |       | | |   | : | :   | | | |:  : |   | |
 29  A E G G G Q N H H E V V K F M D V Y Q R S Y C H P I E T L V  hVPF

 60  D V V S E Y P S E V E H M F S P S C V S L L R C T G C C G D  PlGF
     | :     | | |   | : |   : |   | | | |   | : | | | |   |
 59  D I F Q E Y P D E I E Y I F K P S C V P L M R C G G C C N D  hVPF

 90  E N L H C V P V E T A N V T M Q L L K I R S G D R P S Y V E  PlGF
     |   | | | |   |   : | : | | | : : : | :             |
 89  E G L E C V P T E E S N I T M Q I M R I K P H Q G Q H I G E  hVPF

120  L T F S Q H V R C E C R P - - L R - - - E K M K P E R - C G  PlGF
     : : |   | |   : | | | | |       |       | |   |     |   |
119  M S F L Q H N K C E C R P K K D R A R Q E K - K S V R G K G  hVPF

144  D A V P R - R - - - - - - - - - - - - - - - - - - - - - -  PlGF
         |   |
148  K G Q K R K R K K S R Y K S W S V P C G P C S E R R K H L F  hVPF

...  - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  PlGF

178  V Q D P Q T C K C S C K N T D S R C K A R Q L E L N E R T C  hVPF

...  - - - - - - -                                            PlGF

208  R C D K P R R                                             hVPF
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FIG. 2. Comparison of PlGF and human VPF (hVPF) precursor sequences. Amino acid 1 in this figure corresponds to amino acid −26 in the hVPF published sequence (13). The eight cysteine residues of the PDGF domain are boxed. Vertical lines between two amino acids indicate identity; colons indicate similarity; dash represents a gap introduced to maximize sequence alignment. Putative N-glycosylation sites are underlined.

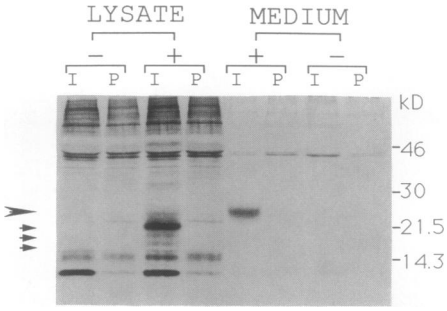

FIG. 3. Immunoprecipitation of ^{35}S-labeled PlGF from COS-1 transfected cells. ^{35}S-labeled lysates or conditioned medium from COS-1 cells transfected with pSVL-PlGF(+) or pSVL(−) plasmids were immunoprecipitated as described using either immune anti-PlGF (lanes I) or preimmune (lanes P) antibodies. Samples were loaded onto a SDS/12.5% polyacrylamide gel under reducing conditions. Large arrow indicates the PlGF corresponding band (≈25 kDa). Small arrows indicate some PlGF immunorelated bands detected only in the COS-1 cell lysates by anti-PlGF antiserum from rabbit. As described in the text, these bands seem to correspond to different N-glycosylated forms of PlGF. Note that in the lysate lanes there are bands with a molecular mass mainly of ≈14 kDa, which are not PlGF related. Positions and marker molecular masses are as follows: ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 21.5 kDa; lysozyme, 14.3 kDa.

N-acetyl-D-glucosamine of the glycosylation core (32), that PlGF is an N-glycosylated protein.

COS-1 transfected cells grown in the presence of tunicamycin (100 μg/ml) produced a protein with a reduced molecular mass (≈15 kDa) (arrow in Fig. 4*A*) that was not efficiently secreted. The treatment of the total proteins from both medium and lysate of COS-1 transfected cells with N-glycosidase F lowered the molecular mass of PlGF at a similar value (arrowhead in Fig. 4*B*).

On the basis of these data, we predicted that PlGF could be mainly located in the endoplasmic reticulum, Golgi apparatus, and cytoplasmic vesicles before being secreted into the medium. This hypothesis was supported by immunofluorescence staining of transfected COS-1 cells expressing PlGF made permeable to the antibodies, in which the protein was localized with an asymmetrical distribution characteristic of the endoplasmic compartment and Golgi apparatus (Fig. 5*A*). When the cells were fixed with paraformaldehyde, PlGF was mainly detected on the cell surface (Fig. 5*B*).

PlGF Is a Dimeric Protein. To understand whether PlGF, like VPF/VEGF, was a dimeric protein (15, 17, 33) with the monomers bound together by disulfide bonds, we performed

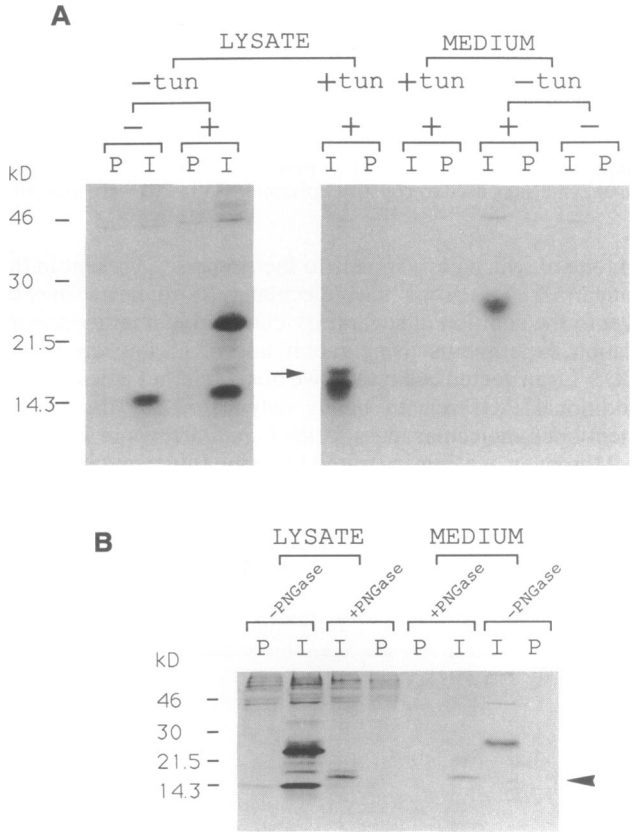

FIG. 4. PlGF is an N-glycosylated protein. Molecular mass marker bands are as in Fig. 3. Rabbit anti-PlGF antibodies were used. Lanes: P, preimmune rabbit serum; I, immune anti-PlGF antiserum. (*A*) Immunoprecipitation of either lysate or conditioned medium from COS-1 cells producing (lanes +) or not producing (lanes −) PlGF. +tun, Cells treated with tunicamycin (100 μg/ml); −tun, cells not treated with tunicamycin. Note that tunicamycin lowers the molecular mass of PlGF (arrow) and inhibits its secretion into the medium. (*B*) Digestion and immunoprecipitation of lysate and conditioned medium from PlGF-producing COS-1 cells with N-glycosidase F (lanes +PNGase). Undigested samples were used as controls (lanes −PNGase). Note that digestion with N-glycosidase F lowers the molecular mass of both totally processed (from medium) and partially processed (from lysate) PlGF protein forms. Additional bands visible in lanes +PNGase are probably due to partial N-glycosidase F digestion.

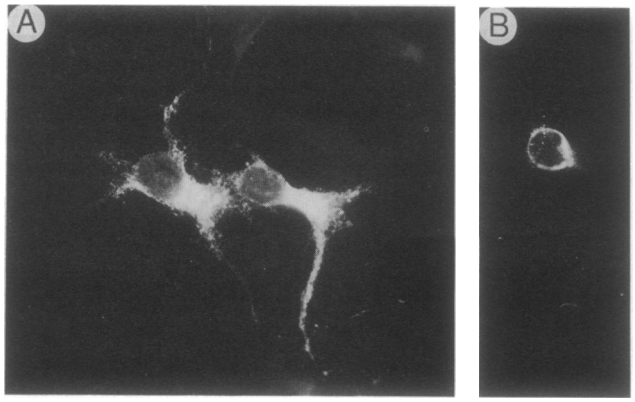

FIG. 5. Detection of PlGF protein in pSVL-PlGF-transfected COS-1 cells after immunofluorescence staining with anti-PlGF antibodies from chicken eggs. Forty-eight hours after transfection, cells were fixed with methanol (*A*) or paraformaldehyde (*B*) and treated with anti-PlGF antibodies from chicken egg yolk. Staining was performed by fluorescein isothiocyanate-conjugated anti-chicken IgG (Sigma).

both immunoblot (Fig. 6*A*) and immunoprecipitation (Fig. 6*B*) experiments using PlGF from transfected COS-1 cells in reducing or nonreducing conditions. In the presence of 2-mercaptoethanol (reducing conditions), the PlGF corresponding band (labeled as monomer in Fig. 6) has a molecular mass of ≈25 kDa, while in nonreducing conditions the molecular mass is doubled (labeled dimer in Fig. 6).

PlGF Is a Mitogen for CPA Endothelial Cells. Among the features common to PlGF and VPF/VEGF, we tested the ability of PlGF to stimulate the growth of the endothelial cells *in vitro*. As shown in Fig. 7, the conditioned medium from pSVL-PlGF-transfected COS-1 cells increased the growth

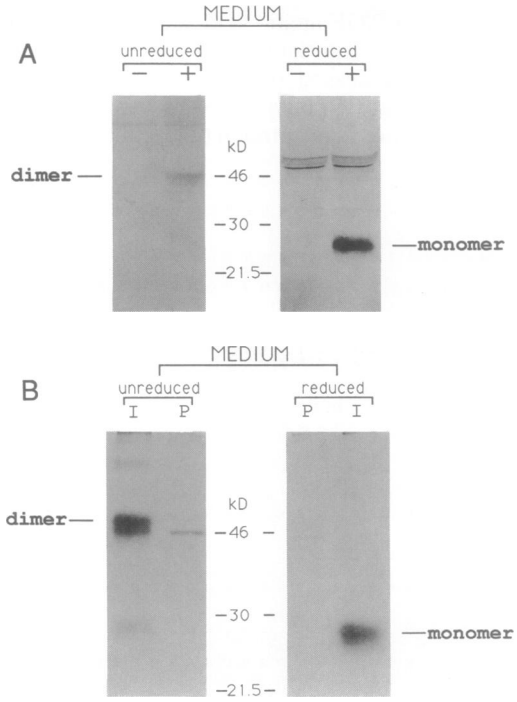

FIG. 6. PlGF is a dimeric protein. Samples were subjected to SDS/12.5% PAGE in reducing or nonreducing conditions. Positions of monomer and dimer PlGF forms are marked. (*A*) Immunoblotting of reduced or nonreduced total conditioned medium from pSVL-PlGF(+) or pSVL(−) COS-1 transfected cells. To perform this test anti-PlGF chicken egg antibodies were used. (*B*) Immunoprecipitation of conditioned medium from PlGF-producing COS-1 cells performed with rabbit preimmune (lanes P) or immune (lanes I) anti-PlGF antiserum.

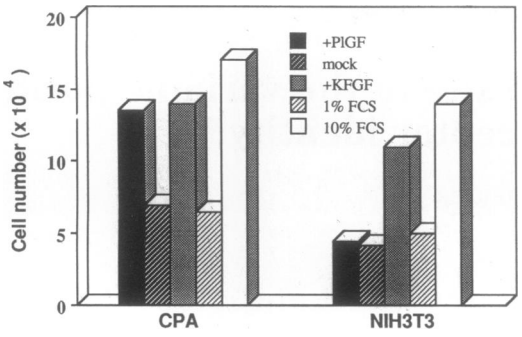

FIG. 7. Mitogenic response of CPA cells to PlGF. CPA and NIH 3T3 cells (4×10^4 cells) were plated in 35-mm-diameter plates in DMEM supplemented with 10% FCS. After 24 hr at 37°C the medium was replaced by fresh medium containing various additions and the cells were incubated for 2 days at 37°C. Histogram gives the number of cells per plate determined after 2 days at 37°C. 1% and 10% FCS, medium supplemented with 1% FCS and 10% FCS, respectively; +PlGF, addition of conditioned medium from COS-1 cells transfected with pSVL-PlGF plasmid at a 1:4 dilution; mock, addition of conditioned medium from COS-1 cells transfected with pSVL plasmid at a 1:4 dilution; +KFGF, addition of conditioned medium from COS-1 cells transfected with p9BKS3A plasmid (22) at a 1:4 dilution.

rate of CPA endothelial cells (ATCC no. CCL207) ≈2-fold compared with the control (conditioned medium from pSVL-transfected COS-1 cells). This effect seems to be specific to endothelial cells because NIH 3T3 cells are not responsive to PlGF (Fig. 7).

DISCUSSION

Human term placenta contains angiogenic activity (34), and one protein with this activity has been isolated from placenta (35). In addition, expression of the platelet-derived endothelial cell growth factor, a recently isolated angiogenic factor, is also detectable in this tissue (9). Thus, human placenta seems to be a source of different angiogenic factors.

In this paper, we describe the cloning of a human cDNA, named PlGF cDNA, corresponding to a gene that is expressed in placenta and in choriocarcinoma cells.

The PlGF cDNA codes for a 149-residue-long protein containing a probable 20-amino-acid-long signal peptide. Thus, PlGF seems to be a secretory protein. We confirmed this hypothesis by testing the PlGF presence in the conditioned medium of transfected COS-1 cells by immunoprecipitation. This and other characteristics are common to VPF/VEGF and PlGF. In fact, in this paper we demonstrated that PlGF, like VPF/VEGF (15, 17, 33), is a dimeric and N-glycosylated protein. Nevertheless, the main feature common to both PlGF and VPF/VEGF is the high amino acid sequence similarity in the PDGF-like region. In fact, the PlGF protein presents a similarity of ≈71% to the PDGF-like domain of human VPF if both the identical and conservative amino acid changes are considered.

Both human and guinea pig VPFs are dimeric and highly basic proteins displaying microheterogeneity with respect to size and charge in different electrophoretic systems (12, 17). The basic region has been localized in the C-terminal region of the human VPF, and it has been suggested that a C-terminal proteolysis and variable glycosylation event might contribute to the heterogeneity of VPF (13). The observation that all the different forms of the guinea pig VPF have the same N terminus because they are all recognized by specific anti-N-terminus antibodies (17) is in agreement with this hypothesis. In addition, all these forms of guinea pig VPF have both permeability-enhancing and growth-promoting activities (17). On the whole, these data indicate that the region of the VPF's activity probably resides in its PDGF-like domain. Thus, because this domain corresponds to the region of maximum similarity between PlGF and VPF we presume that PlGF is also an angiogenic/permeability factor.

Preliminary experiments demonstrate that PlGF can indeed stimulate the growth of the CPA endothelial cell line. However, further studies are necessary to confirm this typical activity normally possessed by a direct angiogenic factor.

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