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Purification and Characterization of a Growth Factor-like Which Increases Capillary Permeability from *Vipera lebetina* Venom

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We have investigated the effect of *Vipera lebetina* venom on capillary permeability and isolated an increasing capillary permeability protein (ICPP) which is devoid of arginine ester hydrolase and phospholipase A2 activities. This protein was purified with a yield of about 0.2% by fast protein liquid chromatography (FPLC) using successively Superose 12, Mono Q, and Mono S columns and by high-pressure liquid chromatography (HPLC) on a C8 reverse-phase column. The purified protein migrated on SDS–PAGE as a band of about 27 kDa under nonreducing conditions and as a band of about 16 kDa under reducing conditions. Chromatography on a C8 column of reduced and alkylated protein yielded a single peak suggesting that this protein is homodimeric. This protein was refractory to Edman degradation chemistry. We used successfully a chemical unblocking involving the incubation of the protein with HCl in anhydrous methanol. The N-terminal amino acid sequence clearly shows considerable similarity to that of vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF).

Snake venoms are rich of proteins and peptides which may be employed to aid our understanding of basic biological mecanisms. Among these proteins, hemorrhagic toxins have been extensively studied (1, 2), and blood capillaries appear to be the main target of these enzymes. They cause a proteolysis of component of the basal lamina of the microvasculature leading to an alteration of the vessel permeability and extravasation (1, 3).

However, many other proteins free of hemorrhagic activity, reported from snake venoms, have been shown to increase capillary permeability. They include bradykinin potentiating factors purified from *Bothrops jararaca* and which are a cytoplasmic membrane peptidases of endothelial cells, responsible both for the conversion of angiotensin I into angiotensin II (4) and for bradykinin degradation (5, 6), kallikrein-like enzymes, described in the venoms of *Trimeresurus mucrosquamatus, Crotalus ruber ruber* and *Agkistrodon caliginosus* which release bradykinin from kininogen (7, 8, 9, 10) and phospholipase A2 purified from *Trimeresurus mucrosquamatus* and which induce a degranulation of mast cells and a release of histamin (11, 12).

In the present study, we report the purification of an homodimeric increasing capillary permeability protein (ICPP) from the venom of *Vipera lebetina.* Sequential homology search of the N-terminal aminoacid sequence determined by automated Edman degradation revealed that this protein have a structural similarity with VEGF and PDGF.

MATERIALS AND METHODS

Materials. Snake venom was collected on ice cooled beakers from *Vipera lebetina* vipers in Pasteur Institute serpentarium and kept as a lyophilized powder. Fast Protein Liquid Chromatography columns were purchased from Pharmacia (Sweden) and reverse phase C8 column (5 μm, 4.6 $\times$ 250 mm) was obtained from Beckman (USA). All reagents used were of analytical grade from commercial sources.

Protein purification. Crude *Vipera lebetina* venom was applied to a Superose 12 prep. grade HR 16/50 column previously equilibred with 0.1 M ammonium acetate pH 8.5 and eluted at 0.5 ml/min on a Fast Protein Liquid Chromatography (FPLC) system (13). The fraction B eluted by gel permeation was loaded onto a Mono Q HR5/5 column pre-equilibred in 0.1 M ammonium acetate pH 8.5 and eluted with a 30 min linear gradient 0.1–0.5 M ammonium acetate pH 8.5 at a flow rate of 1 ml/min monitoring absorbance at 280 nm (14). The first peak fraction was further fractionated on Mono S HR 5/5 column pre-equilibred with sodium acetate 0.05 M pH 5.5. The elution was achieved with Na Cl gradient from 0 to 0.2 M in 30 min and from 0.2 to 0.5 M in 10 min. The ICPP was purified from the main activity peak on a C8 reversed phase HPLC column equilibrated in 0.1% TFA/10% acetonitrile and eluted with a 60 min linear gradient of 10–50% acetonitrile at a flow rate of 1 ml/min monitoring absorbance at 214 nm.

Polyacrylamide gel electrophoresis. The homogeneity and apparent mass of the purified protein and their subunits were determined

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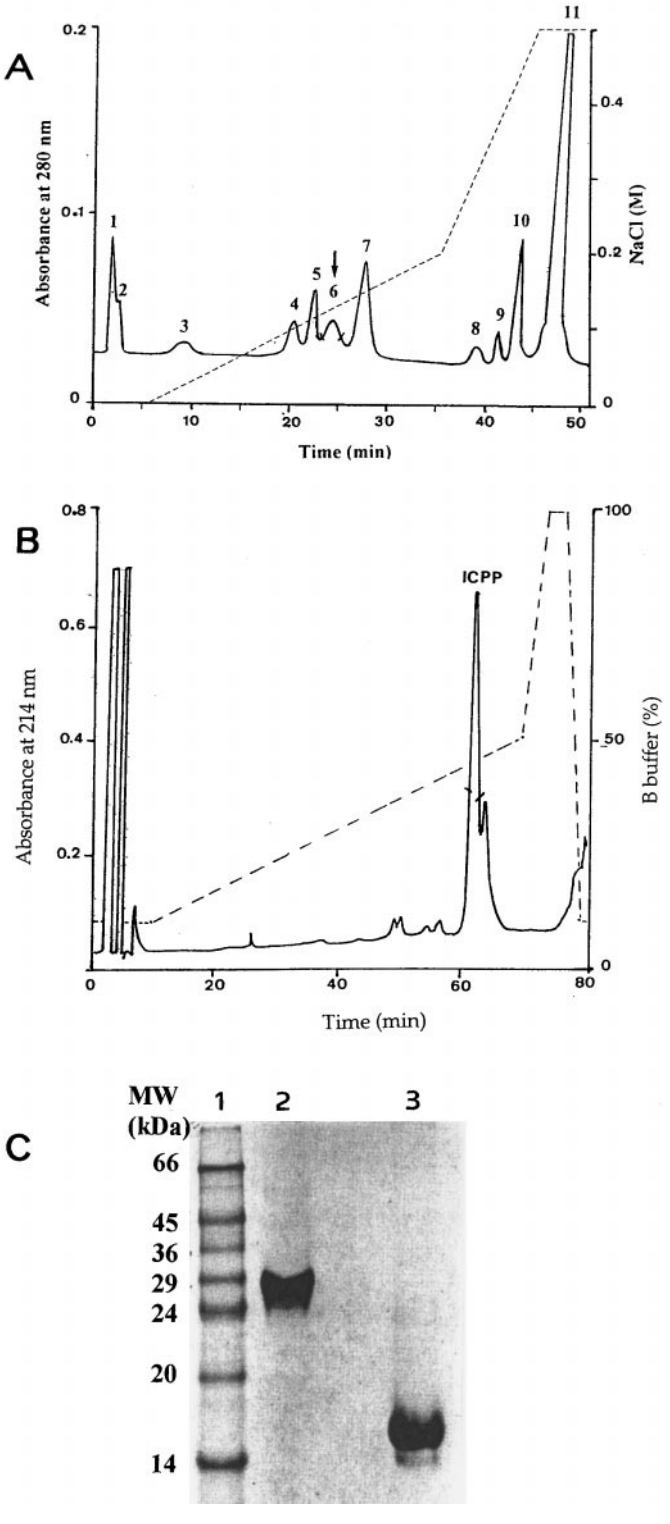

FIG. 1. Purification of ICPP. (A) Chromatography of the fraction B1 (1 mg) obtained after Mono Q on a Mono S column. Elution was performed at a flow rate of 1 ml/min by increasing the NaCl concentration in the Tris-HCl buffer, as indicated on the figure (---). (B) Fraction F6 (0.1 mg) containing the increasing capillary permeability was applied to a reverse phase C8 column. Elution was achieved using a gradient of acetonitrile from 10 to 50% in 60 min. (C) SDS–PAGE analysis of the purified protein. Lane 1: Protein standards; Lanes 2 and 3: ICPP (10 μg) under nonreducing and reducing conditions, respectively.

by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli (15) using 16% polyacrylamide gel with or without prior reduction by β-mercaptoethanol and subsequently visualized by Coomassie blue stain. Commercial medium molecular weight proteins were used as molecular weight standards.

Capillary permeability increasing assay. White Swiss mice (18–20 g) were injected intravenously with 100 μl of a solution of 1% (w/v) Evan's blue dye in phosphate buffered saline (PBS, pH 7.4). After 5 min, varied doses of the crude venom or fractions in 50 μl of PBS were injected intradermally in the mid-dorsal skin depilated 24 h before. After 30 min, the mice were killed and the skins removed. The blue spot where the dye-complexed albumin had penetrated beyond the capillaries indicating the increase of capillary permeability was measured and the area estimated (16). Buffered saline was used as negative control.

Subunit sequencing. Purified ICPP was reduced by incubating for 1 h at 37°C in 6M Gu-HCl, 0.5 M Tris-HCl, 2 mM EDTA, 1.4 μM DTT, pH 7.5 and alkylated by addition of 4-vinylpyridine (9 μM final concentration). Reaction was terminated after 5 min by addition of DTT to a final concentration of 14 μM. The mixture was desalted by chromatography on a reverse phase HPLC on a C8 column as described for protein purification.

Chemical unblocking was performed by incubating the protein in 2 M HCl in anhydrous methanol (17) for 48 h at 25°C. Absence of cleavage of internal peptide bonds which may be caused by this treatment was checked by HPLC on a C8 column.

Sample of unblocked subunit was applied to polybrene-coated glass fiber filters, and its amino-terminal sequence was determined by Edman degradation in an Applied Biosystems 470 A gas-liquid phase microsequencer equipped with an Applied Biosystems 120 A phenylthiohydantoin analyzer.

Protein determination and enzymatic activities assays. Protein concentration was determined by the procedure of Lowry (18) with the folin phenol reagent and with bovine serum albumin as a standard. Phospholipase A2 activity was determined by a colorimetric method using phenol red (19), and arginine esterase activity was assayed with tosyl-l-arginine methyl ester (TAME) by the hydroxamate method (20).

RESULTS AND DISCUSSION

Purification of ICPP to homogeneity was achieved from the crude venom of *Vipera lebetina* in four steps with retention of increasing permeability activity. First by gel filtration in a Superose 12 prep. grade column and anion exchange chromatography column (13, 14), the un-bound fraction in Mono Q was further separated by cation exchange chromatography on a Mono S column (Fig. 1A) and finally fraction 6 obtained from the previous step found to contain the main activity, was loaded on a C8 reverse phase HPLC column (Fig. 1B). Each fraction was assayed for increasing of the capillary permeability using albumin bound with Evan's blue. In normal conditions, the albumin-dye complex does not permeate through the capillary membrane. However, when the capillary is challenged by different amounts of ICPP or fractions containing this

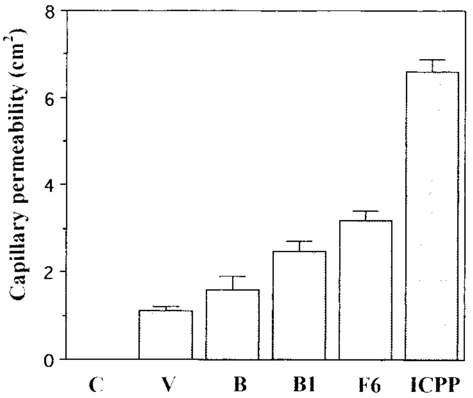

FIG. 2. Activity of the crude venom and of the fractions obtained at the different previous purification steps on capillary permeability on mice. C: negative control, V: crude venom, B1: fraction obtained after Mono Q, F6: fraction obtained after Mono S, ICPP: purified protein after C8 column. An amount of 1 μg was used for each test.

protein its permeability increases and the plasma proteins are suddenly allowed to penetrate the membrane.

The purified component was obtained with a yield of 0.2% from the total venom. SDS–PAGE of the purified ICPP showed a single band having an apparent molecular mass of approximately 27 kDa under nonreducing conditions and a single band of approximately 16 kDa under reducing conditions (Fig. 1C). This result suggests that ICPP is a disulfide linked dimer.

Figure 2 illustrates the activities on capillary permeability at different stages of purification and shows that the purified ICPP seems to be about 6 orders of magnitude more active than crude venom. Enzymatic studies also shows that this protein is free of arginine ester hydrolase and phospholipase A2 activities.

Chromatography on a C8 reverse phase column of reduced and alkylated protein gave a single peak (data not shown). It was concluded that ICPP is homodimeric. Edman degradtion of intact subunit did not allow us to obtain any sequence indicating a blockage at the amino terminus of the molecule. The N-terminal amino acid sequence of this protein was, nevertheless, determined after a successful unblocking assay by HCl and methanol, and the following residues were clearly identified <EVRPFPDVYQRSACQARETLVSILQEYP.

A search for homologous proteins in the BLAST protein database indicated that N-terminal sequence of ICPP showed a significant sequence identity to vascular endothelial growth factor (VEGF) (21), a and b chains of Platalet derived growth factor (PDGF) (22) and vascular permeability factor (VPF) (23). These proteins are structurally related and are potent mitogens, but their target cell specificities and biological properties are different.

VEGF is a heparin-binding growth factor specific for vascular endothelial cells that is able to promote angiogenesis, to cause permeabilization of blood vessels and to induce proliferation of vascular endothelial cells (24, 25). Its activities are mediated by endothelial cell-specific receptor tyrosine kinases, KDR (kinase domain receptor), which triggers the angiogenic response, and Fms-like tyrosine kinase 1 (Flt-1), which function is yet poorly understood (26). VEGF is also a disulfide-linked dimeric protein which may exist in four different homodimeric molecule species by alternative splicing of messenger RNA (21, 27). Each monomer is having 121, 165, 189 or 206 aminoacids, respectively (VEGF121, VEGF165, VEGF189 and VEGF206). The different VEGF isoforms differ in their heparin binding ability. VEGF121 does not bind to heparin, whereas VEGF 165, VEGF189 and VEGF206 do with increasingly greater affinity (28, 29). VPF was identified by cDNA cloning as the same protein as VEGF (21, 23). Whereas, PDGF are dimeric proteins which stimulate growth of a wide variety of cells such as fibroblasts and smooth muscle cell (30) but are inactive on endothelial cells and do not enhance permeability when tested in the Miles permeability assay (16, 31).

Angiogenesis, a process by which new blood vessels form from pre-existing ones, is an essential process in reproduction, development, and wound repair (32, 33, 34). Unregulated angiogenesis is associated with many diseases, such as: arthritis, diabetes, ocular vascularization, proliferative retinopathy, tumor growth, and metastasis (33, 34, 35). Therefore, inhibition of angiogenesis is considered as a major target for development of therapeutic drugs. This concept gained support since a neutralizing murine anti-VEGF antibody, which interact near the KDR and Flt-1 binding sites (36) and SELEX-derived RNA molecules thought to interact specifically with the heparin-binding domain (37) and were shown to inhibit tumor growth *in vivo* (38). Peptides that inhibit binding of VEGF to its receptors, KDR and Flt-1, have been produced using phage display technology and were also shown to antogonize

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CIPP    1                          < E V R P F P D V Y Q R S A C Q A R E T L V S I L Q E Y P
HF      1                          < * * * * * L E * H E * * * * * * * * * * * * * * * * * *
VEGF    1   A P M A E G G G Q N H H * * V K * M * * * * * * Y * H P I * * * * D * F * * * *
PDGFa   71  T K H V P E K R P L P I R R K R S I E E A V P A V * K T * T V I Y E * P R S Q V
PDGFb   47  E L E S L A R G R R S L G S L T I A E P A M I A E * K T * T E V F E * S R R L I
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FIG. 3. Alignment of the N-terminal amino acid sequence of ICPP with those of HF, VEGF, PDGFa and PDGFb. Aminoacid numbering of VEGF is based on the numbering of the mature, secreted polypeptide, whereas the sequences of PDGFa and PDGFb are based on the numbering of cDNA sequences.

VEGF-induced proliferation of primary human umbilical vascular endothelial cells (39).

During the course of determination of primary structure of ICPP, Komori and coll. 1999 (40) described a VEGF-like heparin binding protein (HF) from the venom of *Vipera aspis aspis*. None of the known venom proteins was reported to possess similar characteristics. This protein (HF) was shown to induce mitogenic effect on endothelial cells, hypotensive effect on rats and was able to bind heparin. Figure 3 shows the alignment of the N-terminal aminoacid sequences of ICPP with those of HF, VEGF, PDGFa and b. ICPP shares 86%, 68% and 11% sequence identity to the previous proteins, respectively, and may also shares many of their structural and functional characteristics.

We are currently assessing the full-length amino acid sequence of ICPP and hopefully use this protein to study regulation of angiogenesis and cell proliferation.

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