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Proteolytic processing regulates receptor specificity and activity of VEGF-C

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The recently identified vascular endothelial growth factor C (VEGF-C) belongs to the platelet-derived growth factor (PDGF)/VEGF family of growth factors and is a ligand for the endothelial-specific receptor tyrosine kinases VEGFR-3 and VEGFR-2. The VEGF homology domain spans only about one-third of the cysteine-rich VEGF-C precursor. Here we have analysed the role of post-translational processing in VEGF-C secretion and function, as well as the structure of the mature VEGF-C. The stepwise proteolytic processing of VEGF-C generated several VEGF-C forms with increased activity towards VEGFR-3, but only the fully processed VEGF-C could activate VEGFR-2. Recombinant 'mature' VEGF-C made in yeast bound VEGFR-3 (K_D = 135 pM) and VEGFR-2 (K_D = 410 pM) and activated these receptors. Like VEGF, mature VEGF-C increased vascular permeability, as well as the migration and proliferation of endothelial cells. Unlike other members of the PDGF/VEGF family, mature VEGF-C formed mostly non-covalent homodimers. These data implicate proteolytic processing as a regulator of VEGF-C activity, and reveal novel structure–function relationships in the PDGF/VEGF family.

Keywords: angiogenesis/growth factor/proteolytic processing/VEGF/VEGF-C

Introduction

Angiogenesis, the formation of blood vessels by sprouting from pre-existing ones, is regulated by a balance between positive and negative regulators (Hanahan and Folkman, 1996). Vascular endothelial growth factor (VEGF) belongs to the platelet-derived growth factor (PDGF)/VEGF family and is a major inducer of angiogenesis in normal and pathological conditions (Dvorak *et al.*, 1995; Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996; Ferrara, 1997). The biological effects of VEGF are largely specific for endothelial cells and include stimulation of their proliferation, migration and tube formation, and regulation of vascular permeability (Dvorak *et al.*, 1995; Klagsbrun and D'Amore, 1996; Ferrara, 1997). Another growth factor of the VEGF family, placenta growth factor (PlGF), is expressed predominantly in the placenta; it has minimal angiogenic activity, but is able to heterodimerize with and to modulate the effects of VEGF (Maglione *et al.*, 1991; Park *et al.*, 1994; DiSalvo, 1995; Cao *et al.*, 1996).

VEGF binds to and induces biological responses via two tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk1/KDR), expressed mainly in endothelial cells (see Mustonen and Alitalo, 1995; Shibuya, 1995 for references). PlGF is exclusively a ligand for VEGFR-1 (Park *et al.*, 1994). VEGFR-1 and VEGFR-2 have seven immunoglobulin (Ig)-like loops in the extracellular domain (EC), a single transmembrane region and a tyrosine kinase domain, interrupted by an insert of 60–70 amno acid residues (de Vries *et al.*, 1992; Terman *et al.*, 1992; Shibuya, 1995).

Three novel growth factors strikingly similar to VEGF and PlGF have been identified recently. These factors are the VEGF-B/VEGF-related factor (VRF) (Grimmond *et al.*, 1996; Olofsson *et al.*, 1996a), VEGF-C/VEGF-related protein (VRP) (Joukov *et al.*, 1996; Lee *et al.*, 1996) and c-*fos*-induced growth factor (FIGF) (Orlandini *et al.*, 1996). VEGF-B is most closely related to VEGF and is able to form heterodimers with it (Olofsson *et al.*, 1996a,b). VEGF-C and FIGF are similar in that both have N- and C-terminal extensions flanking a VEGF homology domain. Their C-terminal propeptides contain tandemly repeated motifs with a spacing of cysteine residues typical of Balbiani ring 3 protein (BR3P) (Joukov *et al.*, 1996; Kukk *et al.*, 1996; Lee *et al.*, 1996; Orlandini *et al.*, 1996). Thus, VEGF-C and FIGF comprise a novel subgroup of the PDGF/VEGF family.

The receptors for VEGF-B and FIGF have not yet been identified, while VEGF-C is a ligand for two receptors, VEGFR-3 (Flt4) (Joukov *et al.*, 1996; Lee *et al.*, 1996) and VEGFR-2 (Joukov *et al.*, 1996). VEGFR-3 differs from the two other VEGFRs by being proteolytically cleaved in the extracellular domain into two disulfide-linked polypeptides (Aprelikova *et al.*, 1992; Pajusola *et al.*, 1992, 1993; Galland *et al.*, 1993) and by being expressed in angioblasts of the head mesenchyme and in the veins of embryos, and selectively in lymphatic endothelia thereafter (Kaipainen *et al.*, 1995). The paracrine expression patterns of VEGF-C and VEGFR-3 in many tissues suggest that VEGF-C may function in angiogenesis of the lymphatic vasculature (Kaipainen *et al.*, 1995; Kukk *et al.*, 1996). On the other hand, the ability of VEGF-C to activate VEGFR-2 points to its possible functional redundancy with VEGF.

The VEGF-C precursor is more than twice as large as the mature polypeptide, initially isolated from PC-3 cell culture media (Joukov *et al.*, 1996). This, combined with the unusual structure of the precursor, raised questions

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about the role of its proteolytic processing, possibly affecting receptor specificity, affinity and biological activity. These questions have been addressed in the present study.

Results

Characterization of VEGF-C antibodies and mapping of peptide epitopes in reduced and alkylated VEGF-C polypeptides

To study VEGF-C processing, we first generated antisera recognizing two different regions of the VEGF-C precursor. Antiserum 882 was obtained by immunization with a synthetic peptide corresponding to amino acid residues 2–18 of the N-terminus of mature secreted human VEGF-C [residues 104–120 of the VEGF-C prepropeptide (Joukov *et al.*, 1996); EMBL, GenBank and DDBJ entry X94216]. Antiserum 905 was raised against the N-terminus of the putative VEGF-C propeptide (residues 33–54) (see Figure 3). These antisera and the extracellular domain of VEGFR-3 (R-3EC) were then compared for their ability to bind metabolically labelled recombinant VEGF-C from the conditioned media (CM) of transfected 293-EBNA cells. Both antibodies precipitated VEGF-C forms with molecular masses of 15, 21, as well as a doublet of 29/31 kDa (Figure 1A, lanes 3 and 5, arrows). At higher levels of VEGF-C expression, polypeptides of 43 and 58 kDa were also detected in the immunoprecipitates (Figures 1B and 2). Importantly, both antibodies immunoprecipitated the VEGF-C forms which were able to bind VEGFR-3 (Figure 1A, lane 2). The doublet of 29/31 kDa was the major component of the immunoprecipitates. The 21 kDa band was precipitated by antiserum 905 less efficiently than by antiserum 882, suggesting that a fraction of this form is bound to (a) polypeptide(s) containing also the N-terminal VEGF-C sequence recognized by antiserum 905. Pre-treatment of the antisera with the corresponding peptides used for immunizations abolished their ability to immunoprecipitate the above-mentioned polypeptides (Figure 1A, lanes 4 and 6), indicating that they were specific for VEGF-C.

In order to explore the structure of the VEGF-C peptides further, we compared the abilities of the antisera to bind VEGF-C after reduction and alkylation of disulfide bonds. This treatment prevented the precipitation of the 29 and 43 kDa polypeptides by both antisera and of the 21 kDa form by antiserum 905 (Figure 1B, lanes 1–4). Reduction and alkylation slowed down the migration of the VEGF-C polypeptides in SDS–PAGE, presumably by dissociating intrachain bonds. Therefore, the absence of the 29 kDa form in these conditions could have been due to its co-migration with the 31 kDa component of the doublet. To show that this is not the case, we generated an artificial *N*-glycosylation site in the N-terminal part of VEGF-C by replacing Arg102 with a serine residue, resulting in the NSS(102) peptide (see Figure 3). This mutation slowed down the mobility of the polypeptide normally migrating at 31 kDa and therefore improved the separation of the doublet, thus confirming the above conclusion (data not shown). The mobilities of the 58 and 15 kDa forms were also reduced to 64 and 21 kDa respectively, indicating that these VEGF-C polypeptides contained the appropriate N-terminal peptide of VEGF-C (data not shown). On the other hand, the 21, 29 and 43 kDa forms were not affected by the R102S mutation, suggesting that these polypeptides contain peptide sequences located C-terminally of R102. The specificity of antiserum 905 was demonstrated further by its inability to immunoprecipitate a VEGF-C mutant in which the N-terminal propeptide (residues 32–102) was deleted (ΔN, see Figures 1B and 3). The ΔN polypeptide, immunoprecipitated with the 882 antiserum, migrated in SDS–PAGE with a mobility corresponding to the size of the deletion (~8 kDa) and it was co-precipitated with an equal amount of another pair of polypeptides of 29–32 kDa, which were not recognized by antiserum 882 upon reduction/alkylation of disulfide bonds. These polypeptides were considered to represent heterogenously cleaved/glycosylated C-terminal fragments of the ΔN precursor.

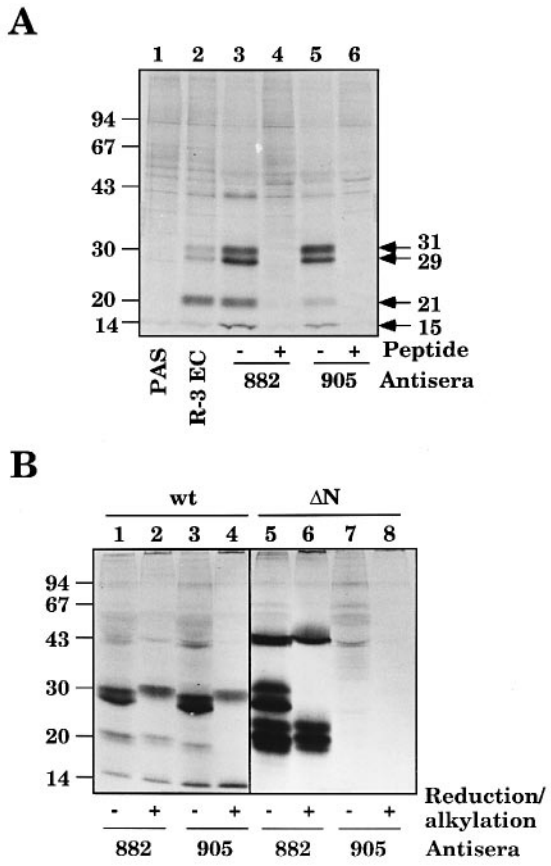

Fig. 1. Recognition of VEGF-C polypeptides by antibodies and VEGFR-3. 293-EBNA cells were transfected with VEGF-C, metabolically labelled, and secreted polypeptides were isolated from the medium with subsequent analysis by SDS–PAGE and autoradiography. (**A**) Wild-type VEGF-C was precipitated from CM using protein A–Sepharose (PAS) only (lane 1), PAS and R-3EC (lane 2), antiserum 882 (lanes 3 and 4) or antiserum 905 (lanes 5 and 6). Lanes 4 and 6 show immunoprecipitation using the antisera pre-treated with the corresponding peptides used for immunizations. R-3EC means recombinant soluble extracellular domain of VEGFR-3. (**B**) The antisera 882 and 905 were used to immunoprecipitate wt (lanes 1–4) or ΔN VEGF-C (lanes 5–8) from non-treated CM (lanes 1, 3, 5 and 7) or from CM treated with dithiothreitol and iodoacetamide to reduce and alkylate disulfide bonds (lanes 2, 4, 6 and 8).

Biosynthesis, dimerization and proteolytic processing of VEGF-C

To analyse the kinetics of VEGF-C biosynthesis and processing, we performed metabolic pulse–chase labelling

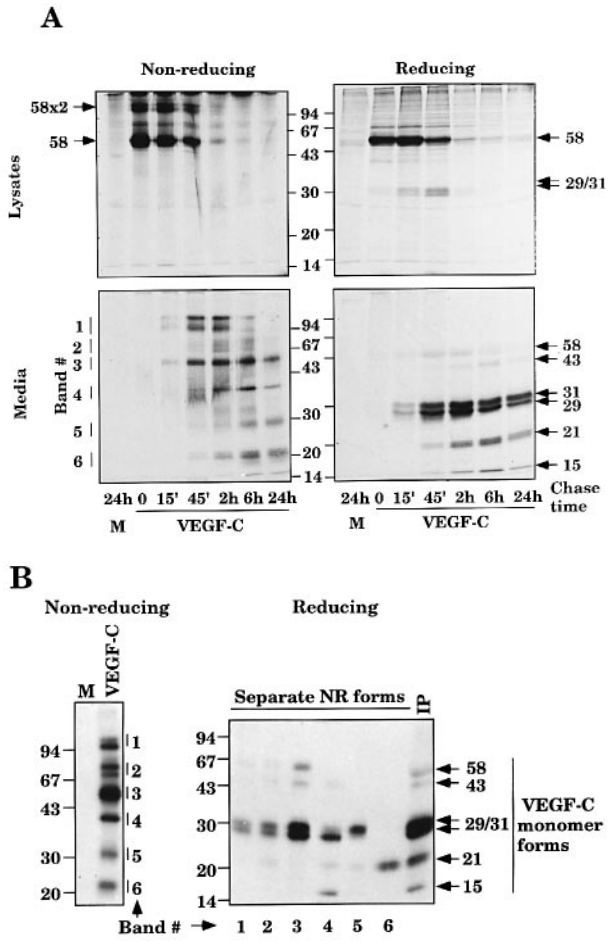

Fig. 2. Biosynthesis, dimerization and proteolytic processing of VEGF-C polypeptides. (**A**) Cells were metabolically labelled for 30 min and then chased in non-radioactive medium for the indicated periods of time. The media and cell lysates were immunoprecipitated with antiserum 882 and analysed by SDS–PAGE in 15% gel in non-reducing and reducing conditions. Mock-transfected cultures (M) were analysed after a 24 h chase period. Numbers on the right and on the upper left panels indicate molecular masses (kDa) of the VEGF-C forms. Band numbers of the lower left panel correspond to those in (B). (**B**) Labelled wt VEGF-C polypeptides were first separated in a non-reducing gel (left panel), excised and subjected to SDS–PAGE in reducing conditions (right panel). The corresponding band and lane numbers are indicated.

experiments with cells expressing recombinant VEGF-C. Analysis of the immunoprecipitated VEGF-C polypeptides after different chase periods in non-reducing and reducing conditions revealed that VEGF-C is first synthesized as a 58 kDa precursor, most of which undergoes dimerization before secretion into the culture medium (Figure 2A, upper panels, arrows '58' and '58×2' in lanes 0–45′). It is cleaved further, forming a 29 and a 31 kDa polypeptide (lanes 0–2 h, arrows 29/31), and rapidly secreted, as only a trace amount of the labelled protein was found intracellularly after a 2–6 h chase period. Most of the secreted VEGF-C was made of disulfide-linked low molecular weight forms at all time points analysed (Figure 2A, lower panels), indicating that proteolytic processing accompanies the secretion of VEGF-C. Proteolytic cleavage was detected in cell lysates at 0 min and in the media after a 15 min chase period, but the resulting chains of 31 and 29 kDa were held together by disulfide bonding (compare lanes 15′–2 h run in reducing and non-reducing conditions). At later chase times, these complexes were cleaved further, with concominant accumulation of a 15 and a 21 kDa polypeptide in reducing conditions (lanes 2–24 h). Importantly, this step of the processing occurs after secretion, as no 15 or 21 kDa forms were detected in the cell lysates (upper panels).

To analyse the composition of the different secreted VEGF-C forms we separated ^{35}S-labelled recombinant VEGF-C polypeptides by SDS–PAGE in non-reducing conditions, excised the polypeptide bands from the gel, reduced the disulfide bonds by treatment of the gel pieces with β-mercaptoethanol and re-analysed the polypeptides in reducing conditions (Figure 2B). The major part of the high molecular weight VEGF-C forms (bands 1–3) gave rise to 29/31 kDa doublets, confirming that the cleaved VEGF-C polypeptides are disulfide-bonded. Only a small fraction of the precursor protein is non-processed or partially processed (products of 58 and 43 kDa in the right hand panel). The low molecular weight components (lanes 4 and 5) contain heterodimerized 15, 21, 29 and 31 kDa polypeptides as well as homodimers of the 31 kDa polypeptide. Interestingly, the monomeric 21 kDa form was also detected (lane 6). The 15 kDa product was disulfide bonded only with the 29 kDa polypeptide (lane 4).

Identification of the proteolytically processed and disulfide-linked forms of VEGF-C

We next used the purified IgG fraction of antiserum 882 to isolate recombinant VEGF-C by affinity chromatography as described in Materials and methods. The purified material contained major polypeptides of 15, 21, 29–30 and 31–32 kDa (data not shown). These polypeptides were subjected to N-terminal amino acid sequence analysis, which gave the sequence NH_2-F(32)ESGLDLSDA-COOH for the 15 and 31–32 kDa polypeptides and the sequence NH_2-A(112)HYNTEILKS-COOH for the 21 kDa form. Because of our inability to obtain an N-terminal sequence for the 29–30 kDa polypeptide, we generated a VEGF-C construct, containing an N-terminal 6×His tag after the signal sequence (see Figure 3, N-His). Polypeptide components of 32 and 29 kDa were obtained after expression and affinity purification of N-His; analysis of the latter polypeptide revealed the N-terminal amino acid sequence NH_2-S(228)LPATL-COOH.

Comparison of the obtained sequences with the sequence of the VEGF-C precursor indicated that polypeptides of 15 and 31 kDa correspond to the N-terminal region of the secreted VEGF-C after cleavage of the signal peptide between Ala31 and Phe32 (Figure 3, arrowhead on the left). The 29 kDa form then represents the C-terminal half of the VEGF-C precursor generated by cleavage between Arg227 and Ser228 (arrowhead on the right). This polypeptide contains one putative *N*-linked glycosylation site and may be cleaved additionally at its C-terminus, as we could not isolate VEGF-C either by using an antiserum against the C-terminal amino acid residues 372–394 or by using the 6×His tag at its C-terminus (data not shown). The 21 kDa form is generated by cleavage of the VEGF-C precursor between Ala111 and Ala112 (grey arrowhead). This cleavage of the recombinant protein thus occurs nine residues C-terminal of the cleavage site located between Arg102 and Thr103, originally described in cultures of

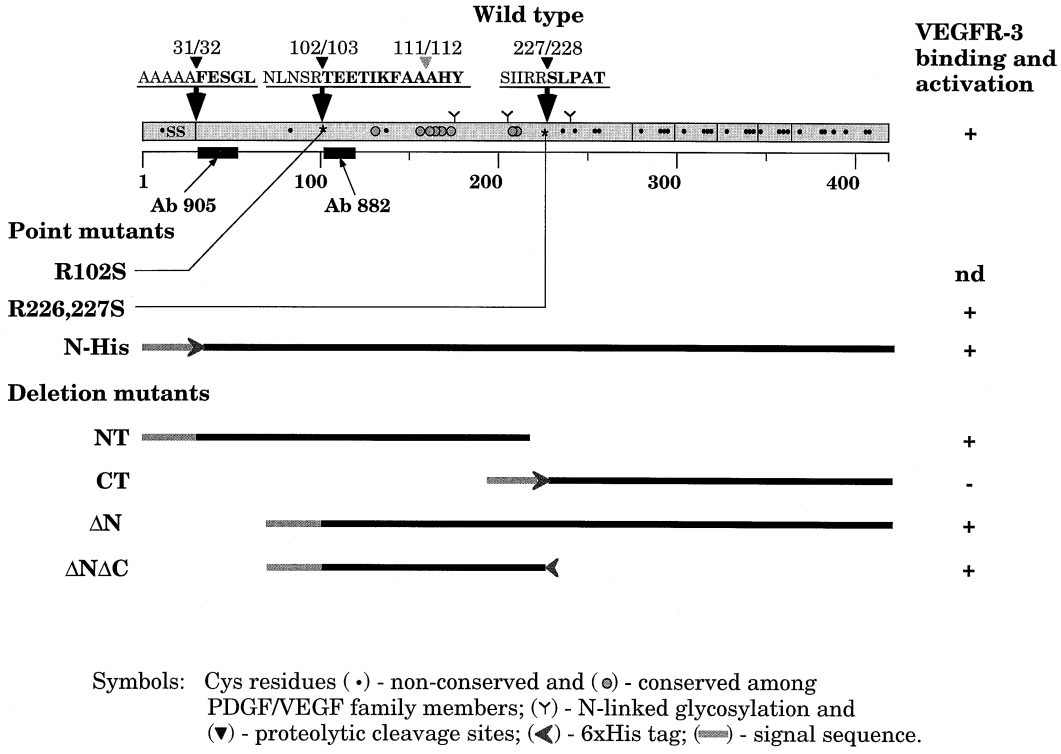

Fig. 3. Schematic structure of the wild-type and mutant VEGF-C forms. The VEGF-C prepropeptide is depicted on the top. The signal sequence and the BR3P motifs are outlined. Peptide sequences adjacent to the proteolytic cleavage sites are also shown. Cleavage sites are indicated by arrows, arrowheads and by numbers of the flanking amino acid residues. Peptide sequences revealed by the N-terminal sequence analysis are marked in bold. The diagram under the box shows the scale in amino acid residues. The epitopes recognized by the antibodies 882 and 905 are marked in the diagram as black boxes. The sites of the point mutations are indicated by asterisks. Other VEGF-C mutants are shown as thick black lines, with the signal sequence marked in grey. The ability of the corresponding construct to bind and to activate VEGFR-3 is indicated on the right (nd, not determined).

PC-3 cells (Joukov *et al.*, 1996). Mature polypeptides of 21 and 31 kDa thus contain the entire VEGF homology domain with all eight conserved cysteine residues and two putative *N*-glycosylation sites.

Taking into account these results and analysis of VEGF-C composition in reducing and non-reducing conditions, one can conclude that the main fraction of processed VEGF-C consists of disulfide-bonded N- and C-terminal parts of VEGF-C precursor cleaved between Arg227 and Ser228 (bands 1–3 in Figure 2A and B). Migration of the proteins in non-reducing conditions suggests that the proteolytic processing occurs gradually. Band 1 presumably contains tetrameric complexes made of two 29/31 kDa dimers linked by disulfide bonds (Figure 2B). Analysis of band 2 suggests that it contains trimers made of the 29/31 kDa dimer disulfide-bonded with the 21 kDa form. It also includes small amounts of 43 and 58 kDa polypeptides. However, the major fraction of the 29 and 31 kDa polypeptides migrates in SDS–PAGE as a disulfide-bonded heterodimer (band 3, compare with bands 1 and 2), while most of the 21 kDa form migrates as a monomer (band 6). Some non-processed monomeric 58 kDa precursor and partially processed 43 kDa polypeptide are also included in band 3. Band 4 is formed mainly by the C-terminal half of the VEGF-C precursor (29 kDa), linked by disulfide bonds with its N-terminal fragment of 15 kDa, and band 5 contains the monomeric N-terminal half of the precursor and a small fraction of the 15 and 21 kDa forms heterodimerized by disulfide bonds. An identical processing pattern was observed when R102S VEGF-C was analysed to improve separation of the 29 and 31 kDa components of the doublet (data not shown).

VEGF-C is processed similarly in different cell types

To exclude the possibility that the observed VEGF-C processing pattern is cell type specific and/or occurs only in cells expressing extremely high VEGF-C levels, we analysed VEGF-C isolated from different transfected and non-transfected cells using the 882 antiserum. The main form of both endogenous VEGF-C, produced by PC-3 cells or HT1080 cells, and of the recombinant VEGF-C expressed in 293-EBNA, COS-7 and HT1080 cells is a doublet of 29/31 kDa. The 15, 21 and 58 kDa VEGF-C forms produced by PC-3 and 293-EBNA cells also had similar mobilities in SDS–PAGE. The proteolytic processing of the VEGF-C precursor in COS cells was less efficient when compared with other cell types, possibly due to a high level of expression or a species difference (data not shown). Taken together, these results indicate that VEGF-C is processed similarly in different cell types.

We further analysed whether the 21 kDa VEGF-C form could be produced by proteolytic cleavage of the 31 kDa form. Serum-free CM was collected from PC-3 cell cultures after various periods, concentrated and analysed by Western blotting using the antiserum 882. As can be seen from Figure 4A, the 21 kDa form accumulated in the medium during cell culturing. A similar product could not be detected in 293-EBNA cells, because cleavage of VEGF-C in these cells occurs more C-terminally (see

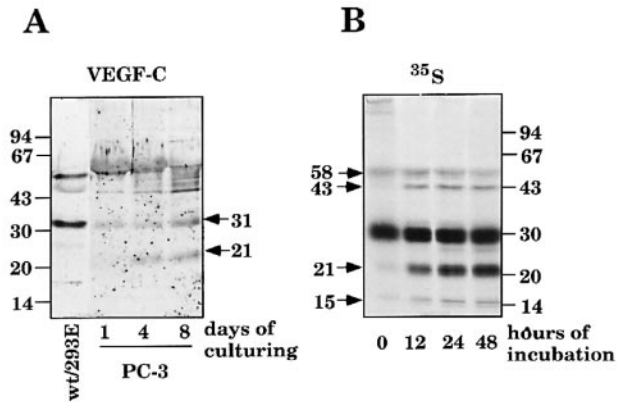

Fig. 4. Proteolytic processing of VEGF-C by secreted protease(s). (**A**) PC-3 cells were cultured for the indicated periods in FCS-free medium, which was then concentrated and analysed by Western blotting and detection using the 882 antiserum. Note that the mature VEGF-C 21 kDa form is detected in PC-3 cells, but not in 293-EBNA cells, transfected with VEGF-C (wt/293E). (**B**) CM from 293-EBNA cells, containing labelled VEGF-C, was incubated with concentrated CM from PC-3 cells for the indicated periods of time, and subjected to immunoprecipitation with antiserum 882. The precipitated material was analysed by SDS–PAGE and autoradiography. Note the accumulation of the mature 21 kDa form during incubation.

Figure 3) and thus deletes about half of the peptide sequence recognized by the antiserum. However, the remaining epitope appears to be sufficient for immunoprecipitation of the 21 kDa form using the same antiserum (Figures 1–3 and 4B). Addition of concentrated CM from PC-3 cells to medium containing ^{35}S-labelled recombinant VEGF-C also caused its proteolytic cleavage, with accumulation of the 21, 43 and 15 kDa products (Figure 4B, arrows), indicating that the protease responsible for VEGF-C cleavage is secreted to the medium. We also observed proteolytic cleavage of the recombinant 31 kDa VEGF-C polypeptide accompanied by simultaneous accumulation of the 21 kDa form upon long-term storage of the CM from transfected 293-EBNA cells (data not shown).

'Recombinantly processed' VEGF-C binds VEGFR-3 and VEGFR-2 with high affinity and induces receptor autophosphorylation

In order to identify and analyse biologically active VEGF-C polypeptides, we generated a panel of deletion mutants of VEGF-C based on the proteolytic processing sites (Figure 3). We found that the ability to stimulate tyrosine phosphorylation of VEGFR-3 and VEGFR-2 depends on the presence of the VEGF homology domain. This conclusion is based on the activating properties of polypeptides encoded by the constructs VEGF-C wt, N-His, NT, ΔN, and ΔNΔC, schematically presented in Figure 3 (data not shown). The construct CT, in which the signal sequence was fused to Ser228 of the C-terminal cleavage site, was expressed efficiently and secreted to the culture medium, but it did not stimulate tyrosine phosphorylation of VEGFR-2 or VEGFR-3 (data not shown). The maximal receptor-stimulating activity corresponded to the 21 kDa form, in which both the N- and C-terminal propeptides were deleted at the proteolytic processing sites or in their close proximity (construct ΔNΔC) (see below).

We next produced the ΔNΔC protein in the *Pichia pastoris* yeast expression system and analysed its ability to bind to and stimulate tyrosine phosphorylation of VEGFR-3 and VEGFR-2 expressed in porcine aortic endothelial (PAE) cells (Pajusola *et al.*, 1994; Waltenberger *et al.*, 1994). Non-transfected PAE cells did not show significant binding of radioiodinated purified ΔNΔC, while specific high affinity binding sites were detected in PAE/VEGFR-3 and PAE/VEGFR-2 cells (Figure 5). The affinities were 135 and 410 pM, respectively, based on Scatchard analysis of the binding data (Figure 5A and B). VEGF-C and VEGF competed with each other for VEGFR-2 binding, VEGF being more efficient in this respect, indicating that the binding involves overlapping sites of the receptor (Figure 5C and D). ΔNΔC, like VEGF, could also be cross-linked to VEGFR-2 on PAE cells (Figure 5F) and it bound to soluble extracellular domains of VEGFR-2 and VEGFR-3. This binding was eliminated by addition of a 30-fold excess of the non-labelled recombinant factor (data not shown). However, ΔNΔC bound neither to the VEGFR-1 extracellular domain (data not shown), nor to the VEGFR-1 expressed in PAE cells (Figure 5E).

Recombinant ΔNΔC, produced both by mammalian and yeast cells, stimulated tyrosine phosphorylation of VEGFR-3 and VEGFR-2 in a dose-dependent fashion at concentrations of 0.2–20 nM (Figure 5G and H and data not shown). This effect was not affected by the presence of the 6×His tag (data not shown). The stimulation of VEGFR-2 was comparable with that of similar concentrations of VEGF. Heparin at 1 μg/ml either did not affect or even decreased binding of ΔNΔC by both receptors (data not shown). Altogether, these data indicate that the proteolytically processed 21 kDa VEGF-C is a ligand and an activator of both VEGFR-3 and VEGFR-2.

Mature VEGF-C has VEGF-like activities

The ability of ΔNΔC to activate VEGFR-2 raised the question of whether it can also induce biological responses characteristic of VEGF. We found that ΔNΔC stimulated the proliferation of bovine capillary endothelial (BCE) cells, although equal stimulation required ~50-fold higher concentrations of VEGF-C in comparison with VEGF (Figure 6A). ΔNΔC, like wt VEGF-C, stimulated the migration of BCE cells in collagen gel, again at higher concentrations when compared with VEGF (Figure 6B). Also, pure recombinant ΔVEGF-C injected subcutaneously into guinea pig skin increased the permeability of blood vessels in a dose-dependent manner (Figure 7A). In this assay, only 4- to 5-fold higher concentrations of ΔNΔC were required compared with VEGF (Figure 7B). Altogether, these data indicate that the proteolytic processing of the VEGF-C precursor generates a biologically active factor which possesses VEGF-like effects on endothelial cells, stimulating their proliferation and migration, as well as the permeability of blood vessels *in vivo*.

Proteolytic maturation affects receptor specificity and activity of VEGF-C

We next addressed the question of whether proteolytic processing affects the ability of VEGF-C to bind and to activate VEGFR-3 and VEGFR-2. In addition to the above-described ΔNΔC, we also generated the VEGF-C R226,227S form in which Arg226 and Arg227, adjacent to the cleavage site, were replaced with serine residues

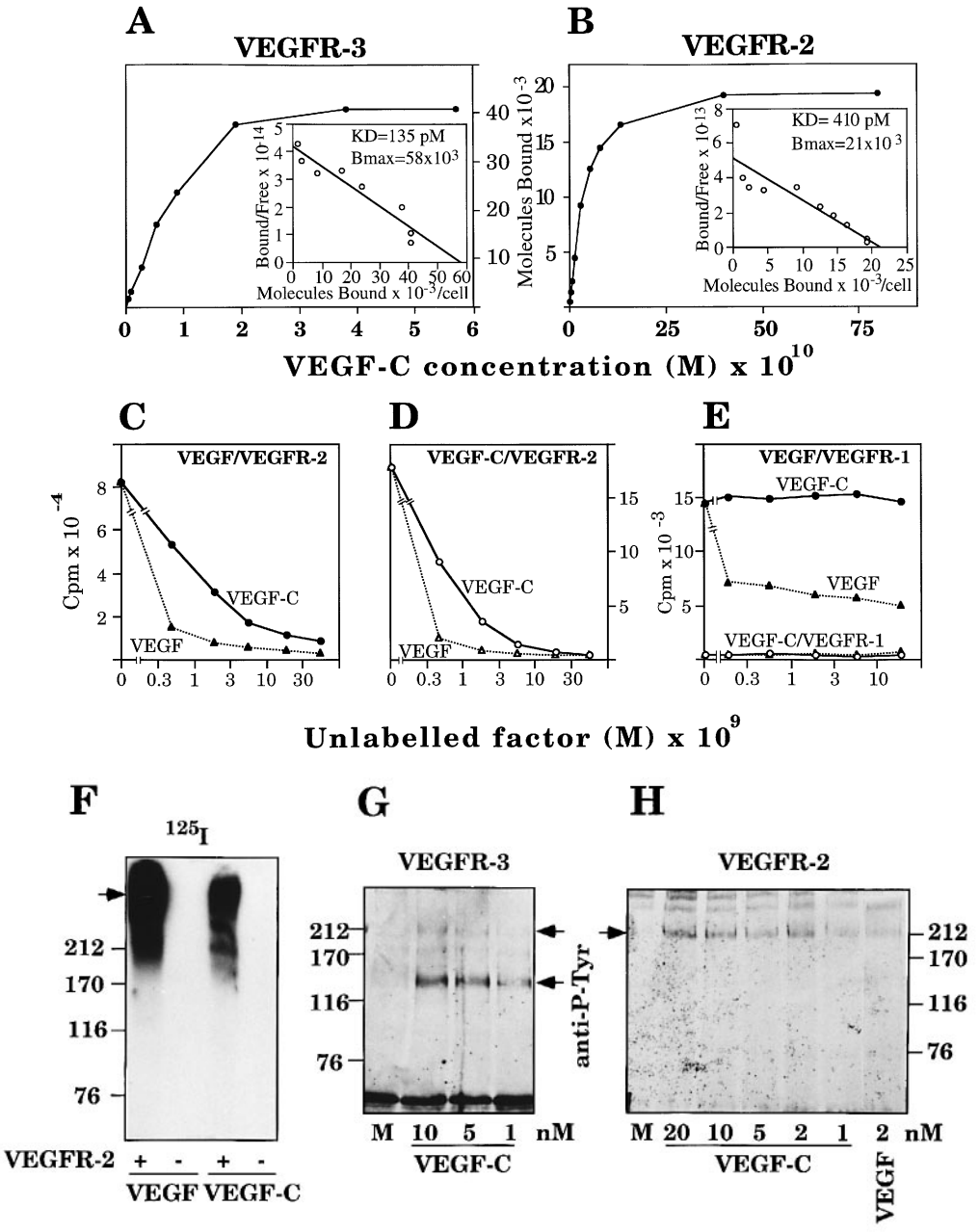

Fig. 5. VEGF-C binds to and activates VEGFR-3 and VEGFR-2. (**A**) and (**B**) Saturation binding curves and Scatchard analysis (inserts) of [^{125}I]ΔNΔC binding to PAE/VEGFR-3 cells (A) and PAE/VEGFR-2 cells (B). (**C**) and (**D**) Displacement of [^{125}I]VEGF (C) or [^{125}I]ΔNΔC (D) from VEGFR-2 by VEGF (triangles) and VEGF-C (circles). (**E**) Displacement of [^{125}I]VEGF (closed symbols) and [^{125}I]ΔNΔC (open symbols) from PAE/VEGFR-1 cells by VEGF (triangles) and ΔNΔC (circles). (**F**) Autoradiogram of VEGFR-2 immunoprecipitates from PAE (–) and PAE/VEGFR-2 (+) cells after cross-linking with [^{125}I]VEGF or [^{125}I]ΔNΔC. The arrow shows the mobility of the major labelled ligand–receptor complex. (**G**) and (**H**) Stimulation of tyrosine phosphorylation of VEGFR-3 and VEGFR-2 by ΔNΔC at different concentrations. Control lanes show analysis of mock-stimulated cells and treatment with 2 nM VEGF. The tyrosine-phosphorylated receptors are marked by arrows. Note the concentration-dependent phosphorylation of VEGFR-2, and of unprocessed 195 kDa and proteolytically processed 125 kDa VEGFR-3 forms in cells treated with ΔNΔC.

(Figure 3). As a consequence, the proteolytic processing at this site was almost completely abolished, as detected by Western blotting using the 882 antiserum (Figure 8A, lane 2). Small amounts of the 31 and 21 kDa polypeptides were, however, found in [^{35}S]R226,227S immunoprecipitates, possibly due to clevage at an alternative site (Figure 9B, lane 1). R226,227S can thus be considered an analogue of the VEGF-C precursor, while wt VEGF-C consists mostly of partially processed 29 and 31 kDa forms, ΔNΔC being an analogue of fully processed, mature VEGF-C (Figure 8A, lanes 3 and 4).

As can be seen from Figure 8B, all processed VEGF-C forms bind to R-3EC, with preferential binding of the 21 kDa form (lanes 2, 5, 7 and 13). Even more striking was the selective binding of the mature 21 kDa form of wt VEGF-C and of ΔNΔC by the VEGFR-2 extracellular domain–alkaline phosphatase fusion protein (R-2EC, lanes 3, 6, 8 and 14). Neither protein A–Sepharose (see Figure

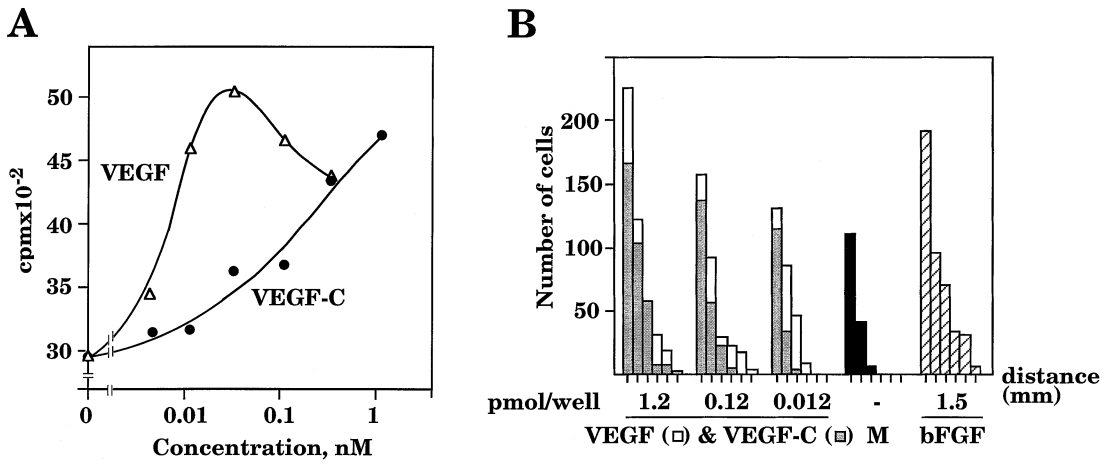

Fig. 6. VEGF-like activity of VEGF-C. (**A**) ΔNΔC stimulation of [^{3}H]thymidine incorporation into DNA of BCE cells. Results of one experiment using different concentrations of the factors are shown. Standard deviations were <10%. (**B**) Migration of BCE cells in collagen gel. The diagram shows the number of cells migrating to six different distances (0.6 μm step) starting from the left (marked by vertical ticks). Analysis employed mock medium (M, black bars) or medium with the indicated amounts of VEGF (open bars), ΔNΔC (grey bars) or bFGF (striped bars).

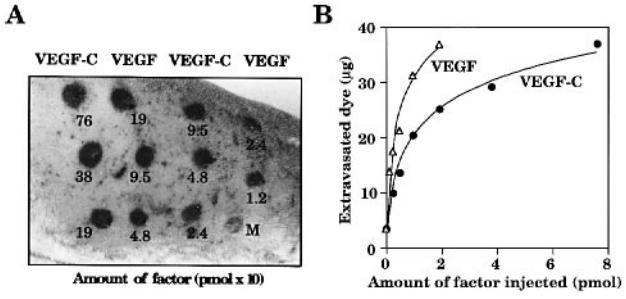

Fig. 7. Comparison of VEGF and VEGF-C in the Miles assay for vascular permeability *in vivo*. (**A**) The indicated amounts of VEGF and VEGF-C were injected intradermally to the back region of a guinea pig. The photograph shown was taken 20 min after the injections. Injection of diluent (PBS/BSA) is marked as M. (**B**) Graphs showing the quantitation of the results of the Miles assay as described in Materials and methods.

1, lane 1) nor protein G–Sepharose alone, or in combination with the anti-alkaline phosphatase antibodies (lanes 10 and 11), bound the 21 kDa form, although very small amounts of the the 58 and 31 kDa VEGF-C forms were bound unspecifically (lanes 8, 9 and data not shown). The specificity of VEGF-C binding was supported by the finding that R-2EC bound VEGF, but not PlGF, while R-3EC did not bind VEGF (data not shown).

Analysis of the receptor-bound material in non-reducing conditions revealed that the 60 kDa polypeptide, which was bound preferentially to R-3EC consisted of disulfide-bonded 29 and 31 kDa heterodimers (lane 13, upper arrow). Most of the 21 kDa polypeptide bound to both R-3EC and R-2EC migrated as a monomer in these conditions (lanes 13 and 14, lower arrow). This finding was most surprising with regard to previously published data concerning other VEGF family members (Maglione *et al.*, 1991; Heldin *et al.*, 1993; Olofsson *et al.*, 1996a; Ferrara, 1997).

We next analysed the ability of the described VEGF-C forms to compete with [^{125}I]VEGF-C(ΔNΔC) for binding to VEGFR-2 and VEGFR-3. As can be seen from Figure 8C, all VEGF-C mutants displaced [^{125}I]VEGF-C from VEGFR-3. The efficiency of displacement was as follows: ΔNΔC>wt>R226,227S, i.e. enhanced binding was obtained upon inclusion of the more mature forms. Recombinant VEGF165 failed to displace VEGF-C from VEGFR-3, but VEGF, ΔNΔC and wt VEGF-C efficiently displaced labelled VEGF-C from VEGFR-2, ΔNΔC being more potent in comparison with wt VEGF-C (Figure 8D). The non-processed R226,227S form showed only weak competition with [^{125}I]VEGF-CΔNΔC, which could be attributed either to its much lower affinity for VEGFR-2, or to the presence of a small amount of the mature forms, cleaved at an alternative site (see above).

Next, we studied the ability of the above-mentioned VEGF-C forms to stimulate tyrosine phosphorylation of VEGFR-3 and VEGFR-2. Stimulation of VEGFR-3 and VEGFR-2 autophosphorylation by the different VEGF-C forms in general correlated with their binding properties and with the degree of proteolytic processing (Figure 8E). ΔNΔC showed a higher activity than wt VEGF-C (lanes 3 and 4), and R226,227S had a considerably weaker effect on autophosphorylation of VEGFR-3, and almost no effect on VEGFR-2 autophosphorylation (lane 2). Finally, the ability of different VEGF-C forms to promote vascular permeability was examined in the Miles assay. CM containing the VEGF-C polypeptides were pre-treated with monoclonal anti-VEGF neutralizing antibodies to eliminate the effect of endogenous VEGF produced by 293-EBNA cells. Although the effect of pure VEGF was neutralized in control experiments, the antibody-treated CM still slightly increased vascular permeability, presumably due to the presence of other permeability factors (Figure 8F and data not shown). CM containing wt and ΔNΔC VEGF-C increased vascular permeability, while the effect of R226,227S CM did not differ significantly from that of CM from mock-transfected cells (Figure 8F). Importantly, identical dilutions of CM were used for these experiments and for the experiments presented in Figure 8C–E. A Western blot analysis of CM using anti-VEGF-C antiserum 882 is shown in Figure 8A to illustrate the relative amounts of the factors present.

Because antiserum 882 did not recognize the mature wild-type polypeptide produced by 293-EBNA cells on a Western blot (see above), metabolic labelling and immuno-

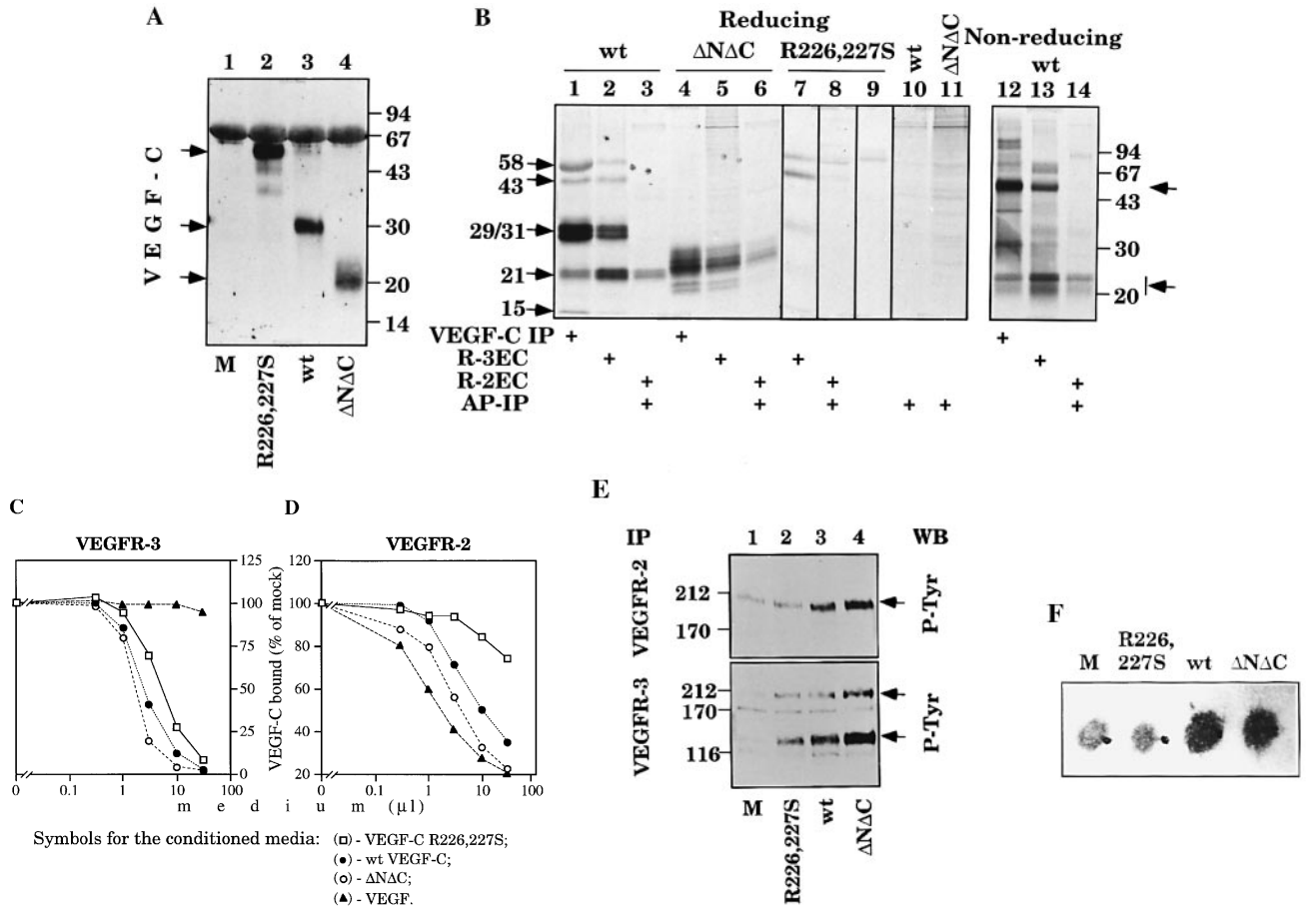

Fig. 8. Proteolytic processing increases the ability of VEGF-C to bind to and activate VEGFR-3 and VEGFR-2. (**A**) Detection of three processed VEGF-C forms by antiserum 882 on a Western blot (arrows). VEGF-C concentrations in CM were adjusted by dilutions with medium from mock-transfected cells. The band at 67 kDa is BSA added as a carrier protein. (**B**) Binding of the various VEGF-C forms by the extracellular domains of VEGFR-3 and VEGFR-2. Metabolically labelled VEGF-C from CM of cells transfected with the indicated constructs was bound to R-3EC (lanes 2, 5 and 7) or to R-2EC (lanes 3, 6 and 8). The receptor extracellular domains were precipitated and analysed by SDS–PAGE. The same amounts of CM were immunoprecipitated (lanes 1 and 4) or treated with PAS only (lane 9). Analysis of material in lanes 1–3 in non-reducing conditions is shown in lanes 12–14. Note that the main fraction of the 21 kDa form bound to the extracellular domains of both receptors does not form disulfide-linked dimers (lower arrow), while most of the 29/31 kDa complexes bound to R-3EC are heterodimerized via disulfide bonds (upper arrow). (**C**) and (**D**) Displacement of [^{125}I]ΔNΔC from the receptors by the VEGF-C forms. PAE/VEGFR-3 (C) and PAE/VEGFR-2 (D) cells were incubated with trace amounts of [^{125}I]ΔNΔC in the absence or presence of different amounts of CM containing the indicated polypeptides, the cells were washed and the amount of bound radioactivity was measured in a γ-counter. Experiments presented in (C), (D) and (E) were carried out using the CM analysed in (A). (**E**) Stimulation of tyrosine phosphorylation of VEGFR-2 (upper panel) and VEGFR-3 (lower panel) by CM from mock-transfected cells (lane 1) and from cells overexpressing the indicated VEGF-C forms. Tyrosine-phosphorylated receptor polypeptides are indicated by arrows. (**F**) Proteolytically processed VEGF-C increases vascular permeability. CM containing ~8 pM of the indicated VEGF-C variants were pre-treated with anti-VEGF neutralizing antibodies and injected intradermally to the back of a guinea pig. The photograph shown was taken 20 min after the injections.

precipitation was carried out to better estimate the relative amounts of each processed form (Figure 9B, lanes 1, 3 and 5). In the experiment presented in Figures 8 and 9, the amount of the 21 kDa polypeptide was approximately one-third of that of the 31 kDa form in the same wt VEGF-C conditioned medium. Taken together, these data indicate that the ability to bind to and to activate VEGFR-3 and VEGFR-2 increases during the proteolytic processing of VEGF-C. Non-processed VEGF-C preferentially binds to and activates VEGFR-3, while the mature 21 kDa VEGF-C form is a high affinity ligand and an activator of both VEGFR-3 and VEGFR-2.

Mature form of VEGF-C consists of non-covalent dimers

Members of the PDGF/VEGF family are active only as dimers. However, as shown above, the proteolytically processed VEGF-C exists mainly as a monomer or a non-disulfide-bonded dimer, which binds VEGFR-3 and VEGFR-2. We were interested in the possibility that dimerization of the processed VEGF-C occurs via non-covalent interactions. Unlike VEGF, which migrates in non-reducing conditions as a dimeric protein of ~44 kDa, most of ΔNΔC migrates as a monomer (Figure 9A). As can be seen from Figure 9B, lanes 6 and 8, about one-half of disuccinimidyl suberate (DSS)-cross-linked VEGF and ΔNΔC migrated as dimers (arrows pointing to lanes 6 and 8 on the right) in reducing conditions. Taking into account that in our conditions ~90% of VEGF migrated as a disulfide-bonded dimer (Figure 9A, lane 1), we conclude that mammalian cells produce ΔNΔC preferentially as a non-covalently bonded dimer (Figure 9A, lane 2 and Figure 9B, lanes 5 and 6). When wt VEGF-C was cross-linked, the amount of the 21 kDa form was

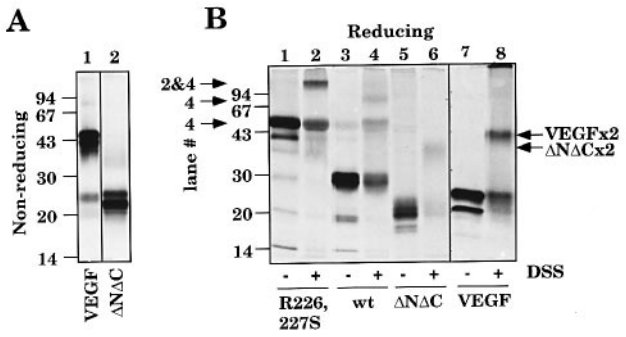

Fig. 9. Mature 21 kDa VEGF-C forms non-covalent dimers. (**A**) Metabolically labelled VEGF165 (lane 1) and ΔNΔC (lane 2) immunoprecipitated from CM and analysed by SDS–PAGE in non-reducing conditions. Note that the majority of VEGF migrates as a dimeric protein, while ΔNΔC migrates as a monomer in these conditions. (**B**) Analysis of the VEGF and VEGF-C forms in CM with (+) or without (–) covalent cross-linking using DSS, followed by immunoprecipitation with the antiserum 882 (lanes 1–6) or with VEGF antibodies (lanes 7 and 8). The numbers and arrows point to the cross-linked dimers and multimers detected in the corresponding lanes. Note that approximately equal proportions of ΔNΔC and VEGF migrate as dimers upon cross-linking (arrows on the right). Note also that complexes of ~ 60 and 80 kDa appear upon cross-linking of wt VEGF-C, and complexes of ~120 kDa are formed when R226,227S is cross-linked (arrows on the left).

considerably decreased (Figure 9B, lane 4), suggesting that it is bound to other polypeptides. Also, additional bands of 60, 80 and 120 kDa appeared in reducing conditions (lane 4, arrows on the left). The first of these apparently represents heterodimers of 29 and 31 kDa forms; the 80 kDa complex is most probably a trimer, consisting of 21, 29 and 31 kDa polypeptides, and the 120 kDa band contains two dimerized VEGF-C precursors, most of which are cleaved at the 227R/228S site. When cleavage between Arg227 and Ser228 was abolished (the R226,227S mutant), no cross-linked complexes of 60 and 80 kDa were detected; instead complexes of ~120 kDa were very prominent, both in non-reducing conditions (data not shown) and upon cross-linking (lane 2). These complexes presumably consist of non-processed VEGF-C dimers linked by disulfide bonds. Despite the fact that we were unable to cross-link the complexes completely, these data, along with the analysis of VEGF-C in reducing and non-reducing conditions, clearly show the co-existence of a variety of its di- and multimeric forms, assembled via disulfide bonding and non-covalent interactions. We also found that recombinant N- and C-termini of VEGF-C were able to form heterodimers when co-expressed in mammalian cells (data not shown), emphasizing the existence of a mechanism for the formation of such dimers in mammalian cells.

Discussion

Proteolytic processing of VEGF-C

Based on the described results, we propose the VEGF-C proteolytic processing model, which is presented schematically in Figure 10. This model resembles the model for the proteolytic processing of PDGF, especially of PDGF-BB (Östman *et al.*, 1988, 1992) in that: (i) the proteolytic cleavages occur after the formation of disulfide-bonded precursor dimers; (ii) both N- and C-terminal propeptides are subject to cleavage; and (iii) a variety of processed forms are secreted. On the other hand, there are several important differences between PDGF-BB and VEGF-C, concerning both their processing and the structure of the mature growth factors.

VEGF-C is released rapidly from cells upon secretion. Upon biosynthesis, two VEGF-C polypeptides, oriented in an anti-parellel fashion, form a dimer linked by disulfide bonds and apparently also by non-covalent bonds. Anti-parallel dimerization is supported by the disulfide bonding of the N- and C- terminal halves (29/31 kDa doublet) of the precursor. Precursor homodimerization is thus followed by the key event in the proteolytic processing—the cleavage between Arg227 and Ser228 dividing the VEGF-C precursor into nearly equal halves. This cleavage site was confirmed by N-terminal peptide sequence analysis and by the R226,227S substitutions, which abolished the cleavage. The N-terminal part of the 31 kDa form contains the VEGF homology domain, and the cysteine-rich C-terminus of the 29 kDa form contains the BR3 motifs. Similarly to PDGF, this processing step occurs in the producer cells, either close to the end of the secretory pathway or at the plasma membrane, because only small amounts of cleaved VEGF-C precursor can be detected in the cell lysates. Most of the secreted VEGF-C is then already cleaved between Arg227 and Ser228, and the resulting polypeptides initially form a tetramer, originating from two precursor polypeptides bound to each other. This processing step is probably conserved in evolution, because the human, mouse and avian VEGF-Cs, as well as FIGF, contain the same amino acid sequence, SIIRRS, surrounding the cleavage site, and a doublet of polypeptides of ~30 kDa is detected in the corresponding immunoprecipitates from the CM of transfected cells (Joukov *et al.*, 1996; Kukk *et al.*, 1996; Orlandini *et al.*, 1996). Morover, most of the VEGF-C secreted by different cell types migrates in reducing conditions as a doublet of 29/31 kDa (data not shown).

The efficient secretion of the R226,227S mutant as well as the presence of small amounts of unprocessed wt VEGF-C precursor in the CM indicate that the intracellular proteolytic cleavage is not a prerequisite for VEGF-C secretion. The C-terminal cleavage of the PDGF-BB precursor occurs in close proximity to the site which corresponds to Arg227 in VEGF-C. Cleavage of high molecular weight VEGF forms by plasmin, with release of diffusible VEGF, also takes place only 10 amino acid residues N-terminal of the VEGF-C cleavage site when VEGF and VEGF-C sequences are aligned (Houck *et al.*, 1992; Keyt *et al.*, 1996a). The proteases responsible for the cleavage of these growth factors might differ, however, because of differences in peptide sequences surrounding the cleavage sites (Östman *et al.*, 1988).

The next step of the proteolytic processing of VEGF-C, which removes the N-terminal propeptide, occurs extracellularly, because the 21 kDa polypeptide was not detected in cell lysates. This differs from the proteolytic processing of PDGF, which occurs exclusively intracellularly (Östman *et al.*, 1992). The 21 kDa form accumulated even upon incubation of cell-free CM, indicating that the cleavage is catalysed by (an) as yet unknown secreted protease(s). Differences in the cleavage sites in cultures of PC-3 and 293-EBNA cells (A111/A112 and R102/

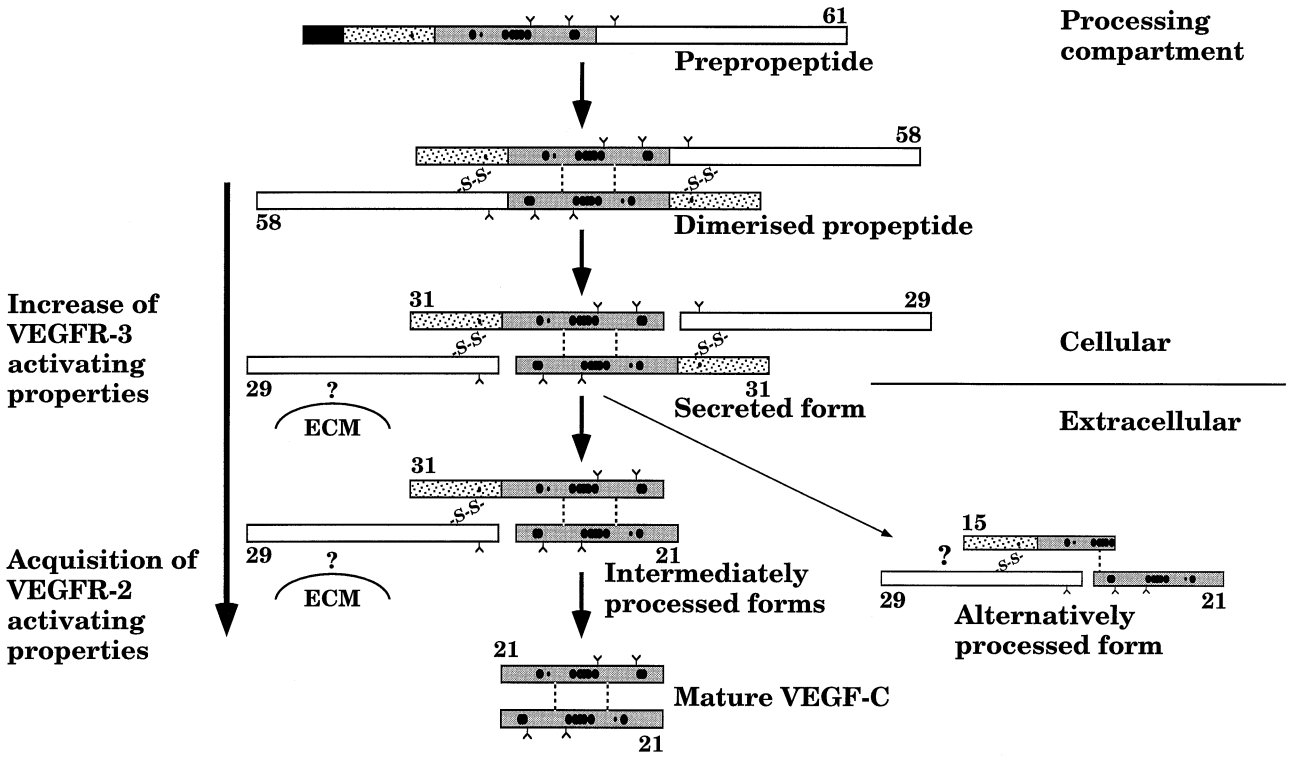

Fig. 10. Schematic model of the proteolytic processing of VEGF-C. The regions of VEGF-C polypeptide are marked as follows: signal sequence, black box; VEGF homology domain, grey box; N-terminal and C-terminal propeptides, dotted and open boxes, respectively. Cysteine residues and putative sites of N-linked glycosylation are shown as in Figure 3; the cysteine residues in the C-terminal propeptide are not marked for clarity. Numbers indicate molecular mass (kDa) of the corresponding polypeptide in reducing conditions. Disulfide bonds are marked as -S-S-; non-covalent bonds as dotted lanes. The hypothetical binding of the C-terminal domain to the extracellular matrix (ECM), and the proposed structure of the alternatively processed VEGF-C are indicated with question marks. The proteolytic generation of a small fraction of disulfide-linked 21 kDa forms is not indicated in the figure. Several intermediate forms are also omitted to simplify the scheme.

T103, respectively) suggest that such proteases are either redundant or that cell type-specific factors determine the exact cleavage sites. This stage of the proteolytic processing occurs in a more gradual fashion, and it finally gives rise to mature VEGF-C, composed of two VEGF homology domains bound by non-covalent interactions.

The small amounts of the shortest identified VEGF-C polypeptide of 15 kDa represent the N-terminal part of the precursor, which binds via disulfide bond(s) with the C-terminal 29 kDa propeptide. The mobility of the 15 kDa form in SDS–PAGE and its recognition by both antisera used in the present study suggest that it contains most of the VEGF homology domain, excluding the first glycosylation site, indicating the existence of an additional proteolytic processing site. Interestingly, this form is very similar to the short splicing variant (clone vh 1.1), reported for VRP (Lee *et al.*, 1996), and thus it may have an analogous, so far unknown function. It is possible that the 15 kDa polypeptide interacts with the 21 kDa form, giving rise to a trimer. It might have an antagonistic activity, competing with the mature ligand for receptors. Small amounts of a secreted 43 kDa form were also detected, but we could not isolate enough of this form to determine its peptide sequence. However, the inability of both VEGF-C antisera to precipitate this form upon reduction/alkylation of disulfide bonds and the correlation of its appearence with the appearance of the 15 kDa form suggest that it might represent the complementary C-terminal part of the VEGF-C precursor after cleavage of the 15 kDa N-terminal part.

Several lines of evidence indicate that mature VEGF-C made by transfected overexpressing cells is a non-covalent dimer. Most of the mature VEGF-C and ΔNΔC migrate at 21 kDa in reducing and non-reducing conditions. Despite this, similar proportions of dimeric molecules (~50%) are detected upon cross-linking the recombinantly produced VEGF and ΔNΔC. Of the various forms, these have the highest affinity for VEGFR-3 and VEGFR-2. The tetra- and trimeric VEGF-C molecules, which were detected upon cross-linking, presumably involve both disulfide bonds connecting the N- and C-terminal parts of separate precursor chains and non-covalent interactions between the two VEGF homology domains (see Figure 10). Subsequent removal of the N-terminal propeptide from both precursors would then explain the formation of the non-disulfide-linked mature VEGF-C dimer.

Absense of interchain disulfide bonds is unusual for the members of the PDGF/VEGF family, in which the second and fourth cysteine residues are involved in anti-parallel interchain disulfide bonds (Andersson *et al.*, 1992; Pötgens *et al.*, 1994). These disulfide bonds are crucial for dimerization and biological activity of VEGF, but are not required for dimerization or mitogenic effects of PDGF-BB (Andersson *et al.*, 1992; Kenney *et al.*, 1994; Pötgens *et al.*, 1994). It has also been shown that the dimer interface in PDGF-BB is sufficient to stabilize the dimer substantially in the absence of a covalent linkage (Oefner *et al.*, 1992). It is possible that such an interaction of the mature polypeptide chains is tighter in VEGF-C than in PDGF-BB. Interestingly, the mature VEGF-C contains an extra cysteine residue at position 137, located between

the first and the second cysteine residue characteristic of the PDGF/VEGF family. This residue is also conserved in mouse and avian VEGF-C (Kukk *et al.*, 1996; A.Eichmann *et al.*, unpublished data) and in FIGF (Orlandini *et al.*, 1996). This residue remains unpaired after cleavage of the N-terminal propeptide, which contains another unpaired cysteine residue.

Properties of the mature VEGF-C

The results with ΔNΔC, which mimics mature VEGF-C, support our earlier observations (Joukov *et al.*, 1996; Kukk *et al.*, 1996) and clearly indicate that proteolytically processed VEGF-C binds to and activates both VEGFR-3 and VEGFR-2. A single class of high affinity sites was observed in PAE/VEGFR-3 cells (K_D = 135 pM) and PAE/VEGFR-2 cells (K_D = 410 pM). These values are of similar magnitude to the affinities reported for the VEGF–VEGFR-2 interaction (Terman *et al.*, 1992; Waltenberger *et al.*, 1994). VEGF-C and VEGF displace each other from VEGFR-2, indicating that the same region of this receptor is involved in binding of both ligands. Surprisingly, none of the three basic residues reported to be critical for VEGFR-2 binding by VEGF (Keyt *et al.*, 1996b) are conserved in VEGF-C, indicating that other residues of VEGF-C are important for its interaction with VEGFR-2. VEGF-C also dose-dependently stimulated autophosphorylation of VEGFR-3 and VEGFR-2 but, in agreement with previously reported data (Lee *et al.*, 1996), we could not detect binding of VEGF-C to VEGFR-1.

Like VEGF, VEGF-C stimulates the proliferation and migration of endothelial cells and increases vascular permeability, albeit at concentrations higher than required for VEGF. These activities are probably mediated through VEGFR-2 activation (Park *et al.*, 1994; Waltenberger *et al.*, 1994). Higher effective concentrations of VEGF-C may depend on its lower affinity for VEGFR-2, and on its inability to bind VEGFR-1, precluding the formation of VEGFR-1–VEGFR-2 heterodimers, which may be required for maximal biological responses to VEGF (Waltenberger *et al.*, 1994; DiSalvo, 1995; Cao *et al.*, 1996; Clauss *et al.*, 1996). The role of VEGFR-2 in the effects of VEGF-C *in vivo* remains to be studied.

The paracrine relationship between the VEGF-C and VEGFR-3 expression patterns in embryos suggests that VEGF-C functions in the formation of the venous and lymphatic vascular systems, where VEGFR-3 is expressed (Kaipainen *et al.*, 1995; Kukk *et al.*, 1996). Our unpublished observations from transgenic mice support such a notion (Jeltsch *et al.*, 1997). However, the redundancy of VEGF-C with VEGF in VEGFR-2-mediated signalling might account for the interesting observations that VEGF –/– mice have delayed endothelial cell differentiation, while in VEGFR-2 –/– mice both haematopoietic and endothelial cell development is aborted, suggesting that (a) VEGFR-2 ligand(s) distinct from VEGF (such as VEGF-C) might play an important role in these processes (Shalaby *et al.*, 1995; Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). Indeed, VEGF-C expression is first detected in day 7 p.c. embryos (Kukk *et al.*, 1996), which is striking, considering the first appearance of the VEGFR-3 mRNA on day 8.5 of gestation (Kaipainen *et al.*, 1995). The question of whether VEGF-C is indeed another factor essential for the development of haematopoietic/endothelial cells will need further studies.

Significance of the proteolytic processing of VEGF-C

Our results demonstrate that during proteolytic processing, VEGF-C acquires the ability to bind to and to activate VEGFR-2, and increases its affinity and activating properties towards VEGFR-3. Many other cytokines and growth factors are also synthesized initially as precursors. These include members of the epidermal growth factor (EGF) family, and the transforming growth β (TGF-β) superfamily, interleukins 1α and 1β, nerve growth factor, hepatocyte growth factor (HGF) and others. Proteolytic processing of TGF-β and HGF precursors is an essential step in the formation of the biologically active ligands (Naka *et al.*, 1992; Vigna *et al.*, 1994; Dubois *et al.*, 1995). Our data indicate that the proteolytic processing of VEGF-C plays a similar role, endowing the mature polypeptide with the ability to activate VEGFR-2. Taking into account the presence of VEGFR-2 in many types of endothelia and the broad expression pattern of VEGF-C, we propose that the biosynthesis of VEGF-C as a precursor prevents unnecessary angiogenic effects, elicited via VEGFR-2, and allows VEGF-C to signal preferentially via VEGFR-3, which is restricted to the venous endothelia during early stages of development and to the lymphatic endothelium during later stages. In certain circumstances, proteolytic processing would release mature VEGF-C, which is able to signal via both VEGFR-3 and VEGFR-2. It is also possible that activation of both VEGFR-3 and VEGFR-2, either as homo- or as heterodimers, is necessary to elicit a complete biological response to VEGF-C. In this case, proteolytic processing might provide a regulatory mechanism which gives the possibility of fine tuning the biological functions of VEGF-C. Also, the extracellular processing step introduces an additional level of regulation of the VEGF-C activity.

An important function of the proteolytic processing of PDGF, and possibly also of certain VEGF isoforms, is to control the bioavailability of the growth factor by removal of the C-terminal propeptide, containing a short stretch of positively charged amino acid residues responsible for the retention of the molecule at the cell surface or in the pericellular matrix (La Rochelle *et al.*, 1991; Houck *et al.*, 1992; Keyt *et al.*, 1996a). The resulting effect is similar to that of alternative splicing, which generates polypeptide variants devoid of the retention domain. VEGF-C is secreted readily into the CM, independently of whether it is processed or not. The isolated C-terminal half of VEGF-C is also released efficiently into the CM, when provided with the VEGF-C signal sequence. In addition, VEGF-C does not bind to heparin, which is known to interact with the basic regions of the long VEGF splice isoforms. The short stretch of basic amino acids, located at the C-terminus of the VEGF-C precursor (residues 372–386), either does not affect its secretion or is proteolytically removed. These data suggest that the bioavailability of VEGF-C is not regulated by the same mechanism as in the case of PDGF and VEGF. The propeptides also do not seem to be essential for the folding, assembly or secretion of VEGF-C homodimers, as the ΔNΔC form

was secreted efficiently as a dimer, and possessed all the tested activities of naturally processed VEGF-C.

The major secreted VEGF-C form contains the C-terminal propeptide which has an unusual structure with tandemly repeated cysteine-rich motifs and is linked via disulfide bonds to the N-terminal propeptide. The possible function of this, apparently by itself inactive C-terminal half of VEGF-C is unknown. Besides its striking similarity to the secretory silk protein (BR3P), the C-terminal VEGF-C propeptide also contains short motifs homologous to the EGF-like domains of other secreted proteins, most importantly of the extracellular matrix components such as fibrillin, laminin and tenascin. All of these proteins are known to participate in protein–protein or protein–cell surface interactions (Apella *et al.*, 1988). This observation, together with increasing evidence that the binding of growth factors to the extracellular matrix is a major mechanism regulating growth factor activity (Taipale and Keski-Oja, 1997), suggests that the secreted VEGF-C, which is proteolytically cleaved at the R227/S228 site, may stay associated with the extracellular matrix via its C-terminal propeptide (Figure 10). The unique organization of the BR3 motifs, which differ from previously known repeated motifs in secreted proteins of vertebrata, might provide additional specificity to the VEGF-C association with the extracellular matrix (Figure 10). Cleavage of the N-terminal propeptide would then release the active VEGF-C not only from the latent state, but also from its association with the extracellular matrix (Figure 10). In fact, some similarity can be seen between structural organization of secreted VEGF-C and TGF-β, with the N- and C- terminal VEGF-C propeptides being functional homologues of the TGF-β latency-associated protein and the latent TGF-β-binding protein respectively. The latter has a domain structure and is similar to fibrillin (reviewed in Miyazono *et al.*, 1994; Taipale and Keski-Oja, 1997). The questions of whether secreted VEGF-C indeed remains associated with the extracellular matrix and which protease is responsible for the proteolytic processing of VEGF-C remain to be answered in the future.

Materials and methods

Cell culture, transfections and metabolic labelling

293-EBNA cells, COS-7 cells, and HT1080 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)–10% fetal calf serum (FCS); PC-3 cells in Ham's F12 medium–7% FCS; PAE-KDR, PAE-Flt1 (Waltenberger *et al.*, 1994) and PAE-Flt4 (Pajusola *et al.*, 1994) cells in Ham's F12 medium–10% FCS. BCE cells (Folkman *et al.*, 1979) were cultured as described in Pertovaara *et al.* (1994). Cell transfections were carried out using the calcium phosphate precipitation method. An equivalent amount of the pREP7 plasmid without insert was used in mock transfections. When used for stimulation experiments, and for detection of VEGF-C expression by Western blotting, the culture medium was changed to DMEM–0.1% bovine serum albumin (BSA) 48 h after transfection and, after an additional 48 h, this medium was collected, clarified by centrufugation, concentrated using Centriprep-10 or Centricon-10 devices (Amicon) and used in the experiments. Metabolic labelling of non-transfected cells and cells transfected with VEGF-C constructs was carried out by addition of 200 and 100 μCi/ml respectively of Pro-mix™ L-[^{35}S] *in vitro* cell labelling mix (Amersham) to the culture medium devoid of cysteine and methionine. After 4 h, the medium was collected or, in some experiments, replaced with DMEM–0.1% BSA, and after an additional incubation for 4 h the media were combined, cleared by centrifugation and used for the immunoprecipitations.

Generation of VEGF-C antisera

Antisera 882 and 905 were generated by immunization of rabbits with synthetic peptides, corresponding to residues 104–120 (NH_2-EETIKFAAAHYNTEILK-COOH) and 33–54 (NH_2-ESGLDLSDAEPD-AGEATAYASK-COOH). The peptides were synthesized as a branched polylysine structure K3PA4 having four peptide acid chains attached to two available lysine residues. The synthesis was performed on a 433A Peptide Synthesizer (Applied Biosystems) using Fmoc-chemistry and TentaGel S MAP RAM10 resin mix (RAPP Polymere GmbH). Cleaved peptides were purified by reverse phase HPLC, dissolved in phosphate-buffered saline (PBS), mixed with Freund's adjuvant and used for immunizations of rabbits at bi-weekly intervals according to standard procedures. Antisera obtained after the fourth and fifth booster immunizations were used in the experiments.

Immunoprecipitation, Western blotting and analysis of receptor autophosphorylation

Receptor stimulation, cell lysis, immunoprecipitation and Western blotting followed previously published procedures (Joukov *et al.*, 1996). Immunoprecipitations of metabolically ^{35}S-labelled VEGF and VEGF-C from CM were carried out using mouse monoclonal anti-human VEGF neutralizing antibody (R&D Systems) and antiserum 882 or 905, respectively. VEGF and VEGF-C bound to antibodies were precipitated using protein G–Sepharose (Pharmacia) and protein A–Sepharose respectively. The peroxidase-conjugated rabbit anti-mouse Ig and swine anti-rabbit Ig (DAKO), diluted 1:1000, and the ECL method (Amersham) were used to detect the polypeptide–antibody complexes on Western blots.

Generation of VEGF-C mutants

VEGF-C mutants were generated using the Altered Sites II *in vitro* Mutagenesis System (Promega). For this purpose, the *Bam*HI fragment of the VEGF-C cDNA from VEGF-C/pREP7 (Joukov *et al.*, 1996) was subcloned in antisense orientation into the pALTER-1 vector. To generate the VEGF-C point mutants, suitable oligonucleotides were synthesized and the mutagenesis procedure was carried out according to the manufacturer's instructions. To generate the N-His construct, an 84mer oligonucleotide was used to introduce the 6×His tag in place of Phe32 (between Ala31 and Glu33). The NT VEGF-C construct was obtained using an oligonucleotide encoding a stop codon instead of Lys214. The deletion mutants were produced by using a loop-out deletion strategy, as described in (Bergman *et al.*, 1995). A 65mer oligonucleotide was used to generate the ΔN VEGF-C construct, in which residues 32–102 of VEGF-C were deleted. In the second round mutagenesis procedure, ΔN VEGF-C and a 52mer oligonucleotide were used to introduce the 6×His tag followed by a stop codon and a *Not*I site immediately after Ile225 to generate ΔNΔC. The CT construct was generated on the basis of the N-His construct. Oligonucleotides (54 and 63mer) were used to introduce *Nco*I sites in the same reading frame, one at the 3′ end of the 6×His tag, and another one at the 5′ end of the sequence encoding the C-terminal part of VEGF-C (starting from Ser228). The resulting construct was subjected to *Nco*I digestion and ligation, giving rise to the construct encoding VEGF-C signal peptide followed by the 6×His tag and the C-terminal half of VEGF-C (additional proline and tryptophan residues were present between the 6×His tag and the C-terminus as a result of introduction of the *Nco*I site in the same reading frame). The mutant constructs in the pALTER vector were digested with *Hin*dIII and *Not*I, subcloned into *Hin*dIII–*Not*I-digested pREP7 and used to transfect 293-EBNA cells.

Strain GS115 of the yeast *P.pastoris* and the expression vector pIC9 (Invitrogen) were used according to the manufacturer's instructions to express ΔNΔC. The VEGF-C sequence was amplified by PCR with a sense primer encoding residues 103–108 and an antisense primer encoding residues 212–215, followed by a 6×His tag. *Eco*RI sites were introduced in the the 5′ and 3′ termini of the sense and antisense primers. The amplified fragment was fused in-frame to the yeast a-factor signal sequence in pIC9.

Purification and N-terminal sequence analysis of VEGF-C

Antibody 882 was employed to purify wt VEGF-C from 1.2 l of CM of transfected 293-EBNA cells by immunoaffinity chromatography. The IgG fraction isolated using protein A–Sepharose (Pharmacia) was covalently bound to CNBr-activated Sepharose CL-4B (Pharmacia) at a concentration of 5 mg of IgG/ml Sepharose resin (Harlow and Lane, 1988). N-His VEGF-C was isolated using Talon™ Metal Affinity Resin (Clontech). Yeast ΔNΔC VEGF-C was purified using Ni-NTA Superflow resin (QIAGEN). No contaminating proteins were detected when 2 μg of the yeast purified ΔNΔC was analysed by SDS–PAGE with subsequent

Coomassie R-250 or silver staining of the gel. The purified material was analysed by electrophoresis, Western blotting and N-terminal amino acid sequence analysis as described earlier (Joukov *et al.*, 1996). An additional sequence obtained during the analysis of the 29–30 kDa polypeptide, NH_2-AVVMTQTPAS-COOH, corresponded to the variable region of the Ig-κ chain, which was present in the purified material due to leakage from the affinity matrix.

Pulse–chase and dimerization studies

Metabolic labelling, immunoprecipitation and pulse–chase analysis of polypeptides were done essentially as described previously (Joukov *et al.*, 1996). To study the composition of the VEGF-C dimers, the labelled polypeptide bands electrophoresed under non-reducing conditions were cut out from the gel, soaked for 30 min in 1× gel-loading buffer containing 200 mM β-mercaptoethanol and subjected to a second SDS–PAGE under reducing conditions. Reduction of the disulfide bonds and alkylation of wt, ΔN and R102S VEGF-C were carried out by incubation of CM in the presence of 10 mM dithiothreitol for 2 h at room temperature with subsequent addition of 25 mM iodoacetamide and incubation for 20 min at room temperature.

For polypeptide cross-linking, DSS (Pierce) was added to the serum- and BSA-free CM at a concentration of 1 mM. After incubation for 1 h, the reaction was quenched by addition of 60 mM Tris–HCl (pH 7.4) and incubation was continued for 30 min. The cross-linked VEGF- and VEGF-C complexes were precipitated using anti-VEGF antibodies and antiserum 882, respectively.

Binding studies using receptor extracellular domains

R-3EC (a kind gift from Dr Katri Pajusola) or R-2EC (Cao *et al.*, 1996) were added to the labelled CM, supplemented with 0.5% BSA and 0.02% Tween-20. A similar amount of CM was used for immunoprecipitation with antiserum 882. After incubation for 2 h at room temperature, anti-VEGF-C antibodies and R-3EC protein were absorbed to protein A–Sepharose, and R-2EC was immunoprecipitated using anti-AP monoclonal antibodies (Genzyme) and protein G–Sepharose. The VEGF-C–receptor complexes were washed three times with ice-cold binding buffer (PBS, 0.5% BSA, 0.02% Tween-20) and twice with 20 mM Tris–HCl (pH 7.4). The same media were precipitated using anti-AP antibody and protein G–Sepharose or with protein A–Sepharose to control possible non-specific absorption.

Analysis of VEGF-C binding to cell surface receptors

Mouse recombinant VEGF164 (a kind gift from Dr Herbert Weich) and pure yeast ΔNΔC were labelled with ^{125}I using the Iodo-Gen reagent (Pierce), and purified by gel filtration on Sephadex G-15 (Pharmacia). The specific activities were 3.5×10^6 c.p.m./pmol and 3.0×10^6 c.p.m./pmol for VEGF and ΔNΔC, respectively. Transfected PAE cells grown on gelatinized 24-well plates (10^5 cells/well) were washed twice with 0.5 ml of binding buffer (Ham's F12 medium, 25 mM HEPES, pH 7.4, 0.1% BSA, 0.1% sodium azide) and incubated for 1.5 h at room temperature in 0.25 ml of binding buffer with increasing concentrations (in saturation analysis) or with a 100 pM concentration of the labelled factor and increasing concentrations of the non-labelled factor (in competition experiments). The cells were then placed on ice, washed three times with ice-cold PBS/0.1% BSA, lysed in 1 M NaOH and counted in a γ-counter. To estimate the non-specific values in saturation binding, the same determinations were done in the presence of unlabelled ΔNΔC.

In order to cross-link the iodinated factors to the cell surface receptors, cells grown on 10 cm gelatinized cell culture dishes were incubated for 1.5 h at room temperature in binding buffer, containing 400 pM of [^{125}I]VEGF or [^{125}I]ΔNΔC. After two washes with PBS, the incubation was continued in PBS containing 0.5 mM of [bis(sulfosuccinimidyl)suberate] (BS^3) (Pierce) for 30 min at room temperature. Then 50 mM Tris–HCl was added to quench the reaction, the cells were washed twice with Tris-buffered saline and lysed in RIPA buffer. The lysates were subjected to immunoprecipitation with VEGFR-2-specific antibodies, and the precipitated material was analysed by SDS–PAGE and autoradiography.

Analysis of VEGF-C biological activity

Mitogenic assays for VEGF-C were carried out by analysis of thymidine incorporation into BCE cells (Olofsson *et al.*, 1996a) and endothelial cell migration assays in the three-dimensional collagen gel as described in Joukov *et al.* (1996). Vascular permeability was determined by the Miles assay (Miles and Miles, 1952). Briefly, depilated guinea pigs were injected intracardially with 20 mg/kg of Evans Blue (Sigma) in 0.5 ml of isotonic saline. The analysed polypeptides were dissolved in PBS and injected intradermally in a volume of 0.1 ml into the back of guinea pigs. In some experiments, the analysed material was pre-treated with 15 μg/ml of anti-human VEGF neutralizing antibody (R&D systems). After 20 min, the animals were sacrificed under anaesthesia, skin at the injection sites was excised and the amount of extravasated dye was quantitated by elution of the dye with 4.0 ml of formamide for 4 days at 45°C and measuring the optical density of the eluate at 620 nm (Udaka *et al.*, 1970). Similar results were obtained in three separate experiments.

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