Signalling properties of an HIV-encoded angiogenic peptide mimicking vascular endothelial growth factor activity

Patrick SCHEIDEGGER*[1], Wolfgang WEIGLHOFER*[1], Stéphanie SUAREZ*, Sandra CONSOLE*, Johannes WALTENBERGER†, Michael S. PEPPER‡, Rolf JAUSSI* and Kurt BALLMER-HOFER*[2]

*Institute of Medical Radiobiology of the University of Zürich and the Paul Scherrer Institute, CH-5232 Villigen-PSI, Switzerland, †Department of Internal Medicine II, Ulm University Medical Center, Robert-Koch-Strasse 8, D-89081 Ulm, Germany, and ‡Department of Morphology, University Medical Center, 1 rue Michel-Servet, 1211 Geneva 4, Switzerland

HIV-1 expresses a multifunctional protein called TAT (*trans*-acting transcriptional activator), the function of which *in vivo* is tightly correlated with the incidence of Kaposi's sarcoma in AIDS patients. TAT is angiogenic and apparently binds to receptors specific for vascular endothelial growth factor (VEGF). Amino acids 46–60 of HIV-TAT, known as the basic peptide, have been shown to be responsible for its functional interaction with VEGF receptors. To characterize further the binding properties of this peptide, its coding sequence was fused to the reading frame of bacterial thioredoxin, allowing the production of large amounts of chimaeric polypeptides in bacteria in a biologically active form. Binding of chimaeric proteins to VEGF receptors was studied *in vitro* in endothelial cell cultures expressing either of the two receptors. Chimaeric thioredoxin proteins carrying the basic domain of TAT bound to both VEGF receptors with affinities similar to those of HIV-TAT or VEGF. Interestingly, these polypeptides competed only partially with VEGF for receptor binding, implying different binding sites for the TAT peptide and VEGF. This suggests that TAT binds VEGF receptors at new sites that might be useful targets for pharmacological intervention during pathological angiogenesis. The thioredoxin/basic-peptide chimaeras are functional agonists that mediate VEGF receptor signalling: (1) they stimulate the growth of endothelial cells; (2) together with basic fibroblast growth factor they cause tube formation of endothelial cells in collagen gels; (3) they induce blood vessel formation on the chicken chorioallantoic membrane; and (4) they activate VEGF receptor kinase and mitogen-activated protein kinase activity.

Key words: angiogenesis, HIV-TAT, signal transduction, VEGF receptor.

INTRODUCTION

Angiogenesis, the process leading to the formation of new blood vessels from existing vessels by sprouting, bridging and intussusception, is regulated by a plethora of growth and differentiation-promoting factors (reviewed in [1,2]). Angiogenic factors are released locally by cells under hypoxic conditions [3]. In a healthy adult mammal, angiogenesis is restricted to sites of vessel injury and to organs of the female reproductive tract, such as the ovaries and the endometrium [4]. Pathological forms of increased angiogenesis have been studied extensively and are well documented for degenerative eye disease [5] and atherosclerosis [6], but also occur during tumour progression (reviewed in [1,7]). Hypoxic tumour cells secrete angiogenic factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), which stimulate endothelial cells in adjacent blood vessels [8].

A complex series of biological effects is required for sprouting of blood vessels during angiogenesis, and VEGF is among the primary factors modulating the behaviour of endothelial cells in this process. It is well established that VEGF induces cell migration [9], stimulates cell growth [10], prevents apoptosis [11–14], promotes the expression of proteases required for invasion into the basal membrane delimiting the layers of smooth muscle and endothelial cells [15,16], and leads to the rearrangement of cell–cell contacts and endothelial cell fenestrae [17,18]. VEGF is a dimeric polypeptide growth factor related to platelet-derived growth factor (reviewed in [19]), and specifically activates endothelial cells upon binding to either of two isoforms of tyrosine kinase receptor, i.e. VEGF receptor-1 [VEGFR-1; also called *fms*-like tyrosine kinase receptor (Flt-1)] [20] and VEGFR-2 [kinase domain receptor (Flk-1/KDR)] [21] (reviewed in [19,22]). Stimulation of endothelial cells by VEGF is absolutely essential for the formation of new blood vessels during fetal development [23–26] and in young animals [27], as demonstrated in VEGF or VEGFR knockout mice. We are interested in the molecular mechanisms responsible for VEGF-mediated signalling that will allow the design of new agonists or antagonists suitable for pro- or anti-angiogenic therapy. Anti-angiogenic therapies directed at tumour-stimulated blood vessels have recently emerged as a promising strategy to destroy growing tumours indirectly by choking oxygen and nutrient supply [7].

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed (e-mail kurt.ballmer@psi.ch).

Abbreviations used: bFGF, basic fibroblast growth factor; BMEC, bovine microvascular endothelial cells; CAM, chicken chorioallantoic membrane; DMEM, Dulbecco's modified Eagle's medium; Erk, extracellular-signal-regulated kinase; FCS, fetal calf serum; Flk/KDR, kinase domain receptor; Flt-1, *fms*-like tyrosine kinase receptor; HUVEC, human umbilical vein endothelial cells; MAP kinase, mitogen-activated protein kinase; PAEC, porcine aortic endothelial cells; RGD region, region that contains the integrin binding site sequence RGD (Arg-Gly-Asp); TAT, *trans*-acting transcriptional activator; Trx, thioredoxin; Trx-bp-loop and Trx-bp-lin, chimaeric proteins comprising Trx with the basic peptide of HIV-TAT ligated into its active-site loop sequence or at its C-terminus respectively; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

Several previous studies have indicated that VEGFRs are also targeted by the TAT (*trans*-acting transcriptional activator) protein expressed by HIV-1-infected cells [28–33]. Located intracellularly, this protein is required for transcription of viral and cellular genes. However, upon secretion via an undefined pathway from infected cells, TAT is also capable of stimulating angiogenesis. This has, for instance, been observed in Kaposi's sarcoma, a condition often observed in AIDS patients. A short basic sequence of 15 amino acids apparently mediates binding to VEGFR-2 [28]. Binding of TAT to this receptor has been shown to be blocked by VEGF, indicating that binding occurs at the same sites as for authentic VEGF [28]. On monocytes, TAT has also been shown to bind VEGFR-1 [34]. Finally, the basic peptide of TAT was shown to stimulate endothelial cell migration in cell culture and to induce angiogenesis *in vivo* in Matrigel implants in mice [31]. From these data, it was concluded that TAT is a functional peptide-mimetic for VEGF, a concept that is also tentatively supported by clinical data [30,35,36].

The fact that this TAT-derived peptide mimics the effects of VEGF on its cognate receptors is puzzling, for several reasons. First, a much smaller binding epitope on the receptor surface is expected to be covered by this low-M_r peptide mimetic as compared with the highly complex dimeric VEGF. Secondly, the functional interaction of receptors with dimeric ligand molecules has been shown to dimerize receptors, followed by activation of the intracellular kinase domain. So far there is no evidence that the TAT basic peptide forms dimers, and it will therefore be interesting to dissect the molecular mechanisms underlying the activity of this VEGF mimetic.

HIV-TAT binds with high affinity to VEGFR-2, while the isolated basic peptide derived from TAT has been shown to be internalized rapidly by cells via an unknown mechanism [37]. This peptide is therefore unsuitable for receptor binding studies on live cells. To investigate its role in VEGFR binding and activation in more detail, we chose to construct two chimaeric polypeptides carrying amino acids 46–60 of TAT either in the active-site loop or at the C-terminus of bacterial thioredoxin (Trx). These polypeptides showed saturable binding to VEGFR-1 and -2. The binding sites for the Trx/basic-peptide chimaera and VEGF are shown to be distinct, as deduced from competition experiments. Both chimaeric proteins had biological activities similar to those of VEGF.

EXPERIMENTAL

Materials

Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics (San Diego, CA, U.S.A.). The cells were grown in endothelial cell growth medium (Clonetics) containing 10% (v/v) fetal bovine serum and the endothelial cell growth supplements provided by the company. The human endothelium-derived permanent cell line EA.hy 926 [38] was obtained from C.-J. S. Edgell (Pathology Department, University of North Carolina, Chapel Hill, NC, U.S.A.) and grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) containing 10% (v/v) fetal bovine serum. Porcine aortic endothelial cells (PAEC) and VEGFR-1- and VEGFR-2-expressing cells were grown in DMEM containing 10% (v/v) fetal calf serum (FCS). Clonally derived bovine microvascular endothelial cells (BMEC) from the adrenal cortex [39], kindly provided by Dr M. B. Furie and Dr S. C. Silverstein (Columbia University, New York, NY, U.S.A.), were cultured in α-modified minimal essential medium (Gibco BRL, Life Technologies, Paisley, Scotland, U.K.) containing 15% (v/v) donor calf serum (Gibco BRL) and antibiotics. Recombinant human bFGF was kindly provided by Dr P. Sarmientos (Farmitalia Carlo Erba, Milano, Italy). Recombinant human VEGF (165-amino-acid homodimeric species) was purchased from PeproTech (Rocky Hill, NJ, U.S.A.). Endotoxin levels were 10.8–13.3 and 11.0–17.0 ng per mg of protein for bFGF and VEGF respectively. Recombinbant canine $VEGF_{164}$ (i.e. VEGF comprising 164 amino acids) was produced as described below [40].

Cloning and expression of canine $VEGF_{164}$

The methylotrophic yeast strain *Pichia pastoris* was used to express both VEGF isoforms. The vector pPICZαA (Invitrogen, San Diego, CA, U.S.A.) has a *Saccharomyces cerevisiae* α-factor secretion signal sequence and was used for expression. The plasmid pCR-Script SK(+) [40] containing the coding region of canine $VEGF_{164}$ was used as a template for subcloning into the eukaryotic expression vector (pPICZαA) by PCR. The following primers were used in the PCR amplification: primer 1, 5′ GCTGAATTCGCTGCGCCTATGGCAGGAGG 3′; primer 2, 5′ ATGATGGTCGACTCATCACCGCCTCGGCTTGACAC 3′. The amplified DNA fragments were purified and digested with *Eco*RI and *Sal*I and ligated with the corresponding vector backbone, followed by transformation into *Escherichia coli* Top10F' (Invitrogen) by electroporation. Positive clones were sequenced to confirm the modification. The plasmid was linearized by digestion with *Bst*XI and electroporated into the yeast strain X-33 for homologous recombination. Electroporated cells were transferred to 1 ml of 1 M sorbitol and plated on to YPDS (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol) plates containing 100 μg/ml Zeocin. For selection, putative multi-copy recombinant colonies were replica-plated on YPDS containing 500, 1000 and 2000 μg/ml Zeocin. Homologous recombination occurs between the 5′ and 3′ ends of the AOX 1 sequence (alcohol oxidase 1 promoter sequence) in the *Pichia* vector. The integration at the correct loci was screened by replica plating on glycerol- and methanol-containing plates. Colonies were allowed to grow for 2 days; they grew normally on glycerol plates, but showed little growth on methanol plates. VEGF was expressed as suggested by the manufacturer.

Scaling up was performed by growing *P. pastoris* in a 3.7-litre fermentor (Bioengineering, Wald, Switzerland). Briefly, fermentation was started with 2000 ml of medium containing 85 g of glycerol and 645 ml of basal salt solution (52 ml/l 85% phosphoric acid, 1.8 g/l $CaSO_4 \cdot 2H_2O$, 28.6 g/ml K_2SO_4, 6.5 g/l KOH and 23.4 g/l $MgSO_4 \cdot 7H_2O$). After sterilization, 8.5 ml of a trace salt solution (20 ml/l 25% sulphuric acid, 65 g/l $FeSO_4 \cdot 7H_2O$, 6 g/l $CuSO_4 \cdot 5H_2O$, 20 g/l $ZnSO_4 \cdot 7H_2O$, 3 g/l $MnSO_4 \cdot H_2O$ and 0.1 g/l biotin) was added and the pH was adjusted to 5.0 by the addition of 20%-satd. ammonium hydroxide. Extensive foaming was controlled throughout the fermentation by the addition of 5% Struktol J673 (Schill & Seilacher, Hamburg, Germany). The fermentor was inoculated with a 50 ml overnight culture in phosphate-buffered medium containing 0.65% yeast nitron base YNB (Difco) and 2% glycerol. Upon exhaustion of the initial glycerol charge, a 50% (w/w) glycerol feed containing 12 ml/l trace salt solution was started at 15 ml/h for 6 h, and a methanol feed containing 12 ml/l trace salt solution was initiated at 5.5 ml/h, to reach a residual methanol concentration of approx. 1% after 6 h. Aeration during fermentation was maintained above 20% air saturation. The cells were harvested 72 h after the methanol feed.

Purification of VEGF

Cell-free medium was collected from the culture and pre-filtered, followed by sterile filtration using a 0.2 μm Nalgene filter system.

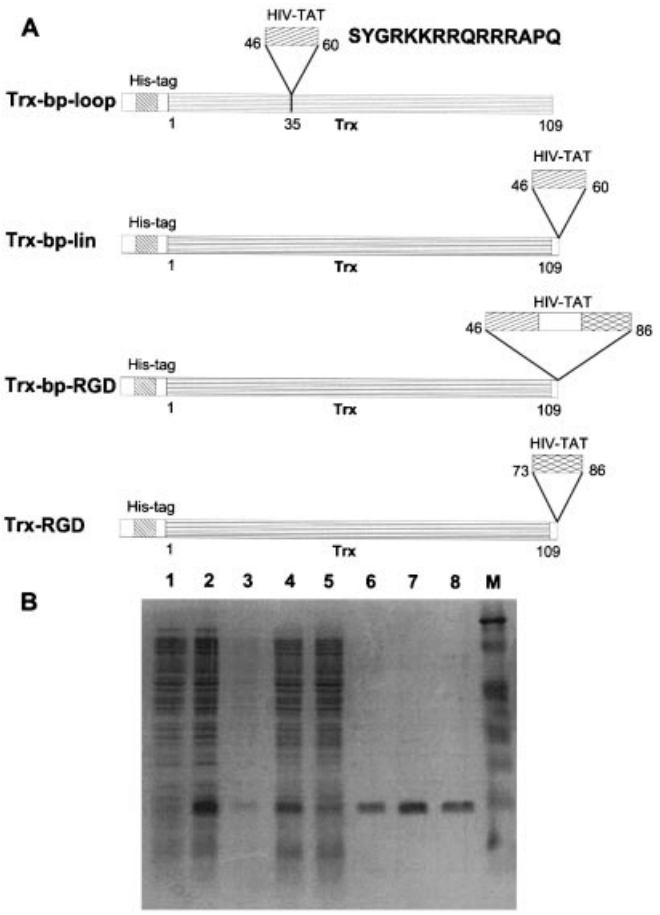

Figure 1 Trx/basic-peptide constructs

(**A**) Schematic representation of the sequences of Trx/basic-peptide chimaeras. All proteins have an N-terminal 6 × His-tag, shown as a diagonally hatched box. The 109-amino-acid Trx sequence is represented as the horizontally hatched region, denoted with Trx starting at position 1 and ending at position 109. Trx-bp-loop contains the basic region of HIV-TAT (amino acids 46–60 of TAT), shown as a diagonally hatched box, inserted at position 35 of the Trx sequence. The other proteins have the short linker sequence Gly-Ser following position 109 of Trx, and carry a C-terminal peptide fusion drawn as differently hatched boxes. Trx-bp-lin contains the basic region of HIV-TAT (amino acids 46–60), Trx-bp-RGD contains the basic region plus the RGD region of HIV-TAT (amino acids 46–86), and Trx-RGD contains only the RGD region of HIV-TAT (amino acids 73–86). (**B**) Purification of Trx-bp-loop. Samples were loaded on to an SDS/15%-polyacrylamide gel and stained with Coomassie Blue G. Lane 1 shows a bacterial culture sample before induction of protein expression with IPTG. Lane 2 shows a sample after 4 h of protein expression. Lanes 3 and 4 show the pellet and supernatant samples respectively after sonication and centrifugation of the bacteria. Lane 5 shows a sample of the lysate after incubation with Ni^{2+}-nitrilotriacetate–agarose beads, and lane 6 shows a sample of the beads after elution of the protein. Lanes 7 and 8 show two samples of purified Trx-bp-loop. The protein migrates below the 20000-M_r band of the low-range protein markers (Bio-Rad) denoted by M.

The medium was concentrated through a YM 30 membrane (Amicon), and VEGF was purified by ion-exchange and gel-filtration chromatography. The concentrated solution was dialysed overnight in 20 mM Hepes, pH 7.4, and loaded on to a Mono Q HR 5/5 column. VEGF was eluted with an NaCl gradient (20 mM Hepes/0–1 M NaCl). The peak fraction was obtained between 0.3 M and 0.6 M NaCl. The fraction was loaded on to a Superose 12 column to separate the dimeric VEGF from salt and from monomeric and multimeric VEGF. The concentration of protein was determined by the colorimetric DC protein assay (Bio-Rad). The purified recombinant proteins were electrophoresed on a 12% (w/v) polyacrylamide gel in the presence of SDS.

Cloning and expression of HIV-TAT

The *E. coli* strain Top10F' was used to express HIV-TAT with the vector pQE30 from Qiagen. This vector contains an optimized promoter/operator element consisting of the phage T5 promoter and two *lac* operator sequences which increase *lac* repressor binding and ensure efficient repression of the powerful T5 promotor. The plasmid pcTAT, obtained from B. R. Cullen (Howard Hughes Medical Institute, Durban, NC, U.S.A.), containing the coding region of HIV-TAT was used as a template for subcloning TAT into the expression vector pQE30 by PCR. The following primers were used in the PCR amplification: primer 1, 5′ GCTGGATCCATGGAGCCAGTAGATCCTAG 3′; primer 2, 5′ GATGAAGCTTTCATTCCTTCGGGCCTG-TCG 3′. The amplified fragments were purified, digested with *Bam*HI and *Hin*dIII and ligated with the corresponding pQE30 fragment, followed by transformation of *E. coli* Top10F' (Invitrogen). Positive clones were sequenced. Expression of Trx constructs and of HIV-TAT was performed in a 3.7-litre fermentor. The bacterial cells were grown until they reached a D_{600} of 0.8–1.2. Expression was induced by adding IPTG to a final concentration of 0.1 mM. After 4 h, the expression was stopped.

Cloning of Trx/basic-peptide chimaera expression plasmids

The Trx coding region plus the stop codon of the plasmid pTrxFus (Invitrogen) was amplified by PCR using the oligonucleotides CTGATCAAGATCTCATATGAGCGATAAAA-TTATTCAC and AGTACAAGCTTTCAGGCCAGGTTAG-CGTCGAG. The resulting fragment was cut with the restriction enzymes *Bgl*II and *Hin*dIII and ligated into vector pQE30 that had been cut with *Bam*HI and *Hin*dIII. This pQE30-Trx plasmid was treated with *Rsr*II, which cuts at the Trx active-site loop sequence. The two annealed oligonucleotides, GTCCGTCC-TATGGCAGGAAGAAGCGGAGACAGCGACGAAGAG-CTCCTCAAG and GACCTTGAGGAGCTCTTCGTCGC-TGTCTCCGCTTCTTCCTGCCATAGGACG, encoding the HIV-TAT basic region (amino acids 46–60), were ligated into this site. The plasmid was named pQE30-Trx-bp-loop.

To fuse the coding sequence of the peptide to the C-terminus of Trx, the Trx coding region without the stop codon was amplified by PCR using the same forward primer as above and the oligonucleotide AGTACAAGCTTTGCAGGATCCGGC-CAGGTTAGCGTCGAG. This generates a *Bam*HI site just downstream of the Trx coding region in the resulting PCR fragment. The fragment was cut with *Bgl*II and *Hin*dIII and ligated into vector pQE30 that had been cut with *Bam*HI and *Hin*dIII. This pQE30-Trx fusion plasmid was cut with *Bam*HI and *Hin*dIII to allow ligation of two annealed oligonucleotides or PCR fragments encoding fusion peptides. The annealed oligonucleotides GATCCTCCTATGGCAGGAAGAAGCGG-AGACAGCGACGAAGAGCTCCTCAATGAA and AGCT-TTCATTGAGGAGCTCTTCGTCGCTGTCTCCGCTTCT-TCCTGCCATAGGAG, encoding the HIV-TAT basic region (amino acids 46–60) plus the stop codon, were ligated into this site to generate the plasmid pQE30-Trx-bp-lin. The annealed oligonucleotides GATCCCCCACCTCCCAATCCCGAGGG-GACCCGACAGGCCCGAAGGAATGAA and AGCTTTC-ATTCCTTCGGGCCTGTCGGGTCCCCTCGGGATTGG-

GAGGTGGGG, encoding the HIV-TAT RGD region [i.e. the region that contains the integrin binding site sequence RGD (Arg-Gly-Asp); amino acids 73–86] plus a stop codon, were ligated in the same way to generate pQE30-Trx-RGD. The plasmid pcTAT (B. R. Cullen) was used as a template for PCR using the oligonucleotides GTACGGATCCTCCTATGGCAGGAAGA-AGC and AGTACAAGCTTTCATTCCTTCGGGCCTGTC-GG to amplify a fragment encoding both the basic region and the RGD region of HIV-TAT (amino acids 46–86) plus a stop codon. The fragment was cut with *Bam*HI and *Hin*dIII and ligated analogously C-terminal of the Trx gene to generate pQE30-Trx-bp-RGD.

Purification of HIV-TAT and Trx chimaeric proteins

Bacteria were collected by centrifugation and lysed by sonication at 4 °C in 6 M guanidine/0.1 M sodium acetate, pH 8.0 (5 ml per g of wet bacterial pellet). The lysate was clarified by centrifugation at 16000 ***g*** for 30 min at 4 °C and purified on Ni^{2+}-nitrilotriacetate–agarose (Quiagen). After binding to Ni^{2+}-nitrilotriacetate, the slurry was washed six times with 6 M guanidine/0.1 M sodium acetate, pH 6.0. The purified protein was refolded on the beads by the addition of 0.25 vol. of PBS every 15 min until the guanidine concentration was below 1 M. The beads were washed twice with PBS and the protein was eluted with 0.2 M EDTA in PBS at 4 °C. The supernatant was dialysed extensively against PBS at 4 °C. The proteins were stored in PBS containing 50 % (v/v) glycerol at −20 °C.

Cell proliferation assay

EA.hy 926 cells were seeded into 24-well tissue culture plates (2.5×10^3 cells/well) in 250 μl of DMEM/10 % (v/v) FCS. After 5 h the medium was removed and the cells were washed with DMEM/0.1 % (v/v) FCS. Proteins were added at a concentration of 2 nM in 250 μl of DMEM/0.5 % (v/v) FCS. After 72 h the cells were treated with trypsin and the cell number was determined in a Coulter cell counter.

In vitro angiogenesis assay

Three-dimensional collagen gels were prepared in 18 mm tissue culture wells as described [41]. Approx. 1×10^5 BMEC in a volume of 0.5 ml of α-modified minimal essential medium containing 5 % (v/v) donor calf serum were seeded on to 500 μl collagen gels in each well. After 3 days, when the cells had reached confluency, medium was changed and donor calf serum was reduced to 2 % (v/v). bFGF or human VEGF (PeproTech), with or without Trx or Trx-bp-loop (a chimaeric protein comprising Trx with the basic peptide of HIV-TAT ligated into its active-site loop sequence; see Figure 1), was added at the concentrations indicated. Medium and compounds were renewed every 2–3 days. Cultures were photographed after 4 or 7 days. Invasion was quantified from three randomly selected fields per well, each measuring 1 mm × 1.4 mm, by counting the total length of all cells which penetrated into the underlying gel either as single cells or in the form of cell cords [47]. Results are from at least four experiments, i.e. at least 12 microscopic fields per condition (three measurements per experiment).

Radioiodination and binding studies

Recombinant $VEGF_{164}$ and Trx-bp-loop were radioiodinated by the Iodogen method. Samples of 20 μg of $VEGF_{164}$ or 6 μg of Trx-bp-loop were treated with 1 mCi of carrier-free $Na^{125}I$ (Amersham Pharmacia Biotech) in a tube precoated with 50 μg or 1 μg respectively of Iodogen (Pierce, Rockford, IL, U.S.A.) for 10 min at 0 °C. The reaction was terminated by separating the protein solution from Iodogen. The protein-associated radioactivity was separated from free iodine on a PD10 gel-filtration column (Amersham Pharmacia Biotech) in the presence of 0.5 % (w/v) BSA in PBS. The specific radioactivities of $VEGF_{164}$ and Trx-bp-loop were 9×10^4 c.p.m./ng and 2.5×10^5 c.p.m./ng respectively.

HUVEC and PAEC were grown overnight to confluency in 24-well tissue culture plates. Binding assays were carried out in triplicate using ^{125}I-VEGF (0–10 ng/ml) or ^{125}I-Trx-bp-loop (0–5 ng/ml). To determine non-specific binding for each data point, radiolabelled peptide was added together with a 200-fold excess of unlabelled protein. Binding assays were performed at 4 °C for 1 h. The cells were then washed with 0.5 % BSA in PBS and lysed with 1 M NaOH. The radioactivity associated with the cells was determined in a Beckman γ-radiation counter. The data were plotted according to Scatchard [42], and a linear regression analysis (SigmaPlot) of the data points was performed to determine the dissociation constant (K_d, derived from the slope of the linear regression curve) and the receptor number per cell (derived from the intersection of the linear regression curve with the x-axis). For competition assays, cells were incubated with 10 ng/ml ^{125}I-labelled $VEGF_{164}$ or with 5 ng/ml ^{125}I-labelled Trx-bp-loop, with or without the indicated amount of unlabelled protein, for 2 h at 4 °C, followed by washing and cell lysis. In all experiments each data point was determined in triplicate.

VEGFR-2 and mitogen-activated protein (MAP) kinase assays

Kinase assays for VEGFR-2 and the extracellular-signal-regulated kinase (Erk) branch of MAP kinases were performed in PAEC or HUVEC as described [43,44]. Immunoprecipitation of VEGFR-2 was performed with a polyclonal rabbit antiserum obtained from Dr A. Kazlauskas (The Schepens Eye Research Institute, Harvard Medical School, Boston, MA, U.S.A.), and Erk1 and Erk2 were immunoprecipitated with rabbit antibodies obtained from Dr D. Fabbro (Novartis, Basel, Switzerland). SDS/PAGE gels were treated with 1 M KOH for 60 min at 55 °C to decrease background in VEGFR-2 assays.

RESULTS

Chemically synthesized basic peptide encompassing amino acids 46–60 of HIV-TAT was radiolabelled with ^{125}I and used for VEGFR binding assays on HUVEC. The material was unspecifically taken up by cells in an unsaturable way, and no specific binding could be detected (results not shown). This was reminiscent of earlier studies that showed that this peptide was able to be translocated efficiently across the plasma membrane via an unknown mechanism [37]. We therefore chose to construct chimaeric proteins that carry this peptide sequence in a structurally restricted conformation in the context of a carrier protein, thus reflecting its arrangement in HIV-TAT. Amino acids 46–60 of HIV-TAT were expressed either in the active-site loop (Trx-bp-loop) or at the C-terminus (Trx-bp-lin) of bacterial Trx. All Trx constructs were tagged with six histidine residues at the N-terminus for easy purification (Figure 1A). Chimaeric Trx/basic-peptide proteins were expressed in *E. coli* and purified and labelled as decribed in the Experimental section. An example of the purification for one of these Trx fusion proteins, Trx-bp-loop, is shown in Figure 1(B).

Next we compared the binding properties of canine $VEGF_{164}$ produced in *P. pastoris* on HUVEC, a primary endothelial

Table 1 Binding of $VEGF_{164}$ to HUVEC and PAEC

Binding studies with radioiodinated VEGF were performed as described in the Experimental section. Data shown are averaged from three separate experiments, ±S.E.M.

	High-affinity receptors		Low-affinity receptors	
Cell line	K_d (pM)	Receptor number per cell	K_d (pM)	Receptor number per cell
HUVEC	28±4	(34±4.5)×10^3	420±180	(110±11)×10^3
PAEC-Flt-1	22±5	(36±8)×10^3	–	–
PAEC-KDR	–	–	500±150	(110±15)×10^3

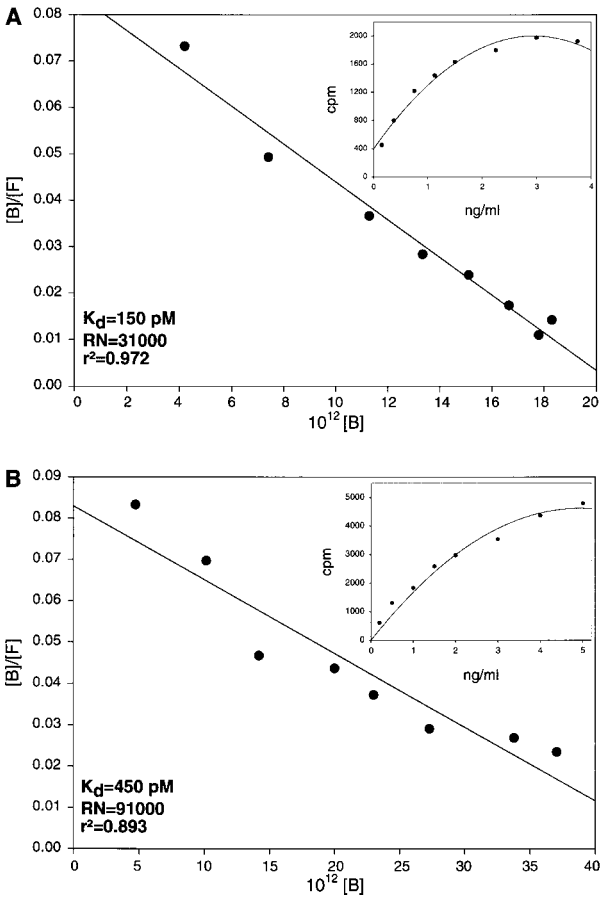

Figure 2 Binding of Trx-bp-loop to endothelial cells

Representative binding curves (insets) and Scatchard plots of Trx-bp-loop bound to PAEC-Flt-1 (**A**) and PAEC-KDR (**B**) cells are shown. Values of dissociation constant (K_d) and receptor number per cell (RN) were calculated using Sigma Plot linear regression analysis of the data; the correlation coefficient (r^2) for the analysis is indicated. Binding studies were performed in triplicate; the S.E.M. of each data point was < 8.2%.

cell expressing both VEGFRs (Table 1). The apparent dissociation constants were approx. 28 pM for binding to VEGFR-1 and 420 pM for binding to VEGFR-2, in agreement with published data [45]. The number of receptors expressed per cell was also similar to values published previously. We also determined the binding properties of $VEGF_{164}$ on PAEC, which lack endogenous VEGFRs and which were transfected with plasmids encoding either VEGFR-1 or VEGFR-2 [45]. The observed affinity constants for $VEGF_{164}$ binding were similar to those observed on HUVEC (Table 1).

The chimaeric Trx construct expressing the basic TAT peptide sequence in the catalytic loop (Figure 1A) was studied next. Trx-bp-loop bound specifically to PAEC expressing VEGFR-1 (Figure 2A) or VEGFR-2 (Figure 2B), with K_d values of 150 and 450 pM respectively. The receptor numbers on PAEC were similar to those seen for VEGF, i.e. approx. 30000 and 90000 receptors for VEGFR-1 and VEGFR-2 respectively. In all assays, binding was blocked efficiently with unlabelled ligand, indicating that interaction with the receptor was specific and that iodination did not change the binding properties of the ligands. These data show that the basic peptide derived from HIV-TAT interacts specifically with both VEGFRs, even when expressed as a fusion protein with bacterial Trx. Trx lacking the TAT sequence did not bind to VEGFR-expressing PAEC. Finally, untransfected PAEC showed no specific binding of any of the Trx/basic-peptide chimaeric proteins (results not shown).

We next asked whether our Trx/basic-peptide chimaeras bound to the same sites on VEGFRs as VEGF. Figures 3(A) and 3(B) show representative binding assays with radiolabelled $VEGF_{164}$ and a series of unlabelled competitors. While VEGF efficiently blocked binding of ^{125}I-VEGF to both VEGFR-1 and VEGFR-2, the Trx-bp-loop chimaera only partially blocked binding of VEGF. Trx alone and a chimaeric Trx protein carrying an integrin-specific RGD sequence derived from HIV-TAT (amino acids 73–86) in place of the TAT basic peptide sequence (see Figure 1) did not compete with VEGF for either of the two receptors. The reverse experiment, in which ^{125}I-labelled Trx-bp-loop was competed with unlabelled VEGF, also showed that Trx/basic-peptide chimaeras and VEGF bound to distinct sites on both VEGFRs (Figures 4A and 4B). Trx-bp-loop and Trx-bp-lin competed efficiently for binding to both receptors, suggesting that the basic peptide is similarly exposed in both fusion proteins. The Trx/RGD chimaera did not block binding of the Trx/basic-peptide chimaeras, in agreement with earlier findings that RGD peptides bind to distinct isoforms of the integrin family of cell surface receptors, but not to VEGFRs. In conclusion, these data show that the basic peptide sequence present in HIV-TAT interacts with both VEGFRs. Binding of this peptide to VEGFRs is highly specific and involves interaction with receptor sites that are, at least partially, distinct from VEGF binding sites. The fact that the basic peptide and VEGF show weak competition in binding assays might be indicative of allosteric interaction between binding sites specific for these two ligands.

We next studied the mitogenic activity of the Trx/basic-peptide chimaeric proteins in an immortalized endothelial cell line derived from HUVEC, EA.hy 926 [38]. As shown in Table 2, Trx-bp-lin and Trx-bp-loop triggered cell growth to a similar extent as did full-length HIV-TAT protein or $VEGF_{164}$, while Trx alone was completely inactive. When cells were first starved for more than 4 days in < 0.1% serum, a high rate of apoptosis was observed. Under these conditions VEGF or the Trx/basic-peptide chimaera did not stimulate cell growth, but behaved as survival factors (results not shown).

We have reported previously that bFGF and VEGF induce BMEC to invade three-dimensional collagen gels, within which they form capillary-like tubes [46,47]. The effect induced by bFGF is maximal after 3–4 days, whereas at least 7–10 days are required to see a maximal effect with VEGF [48]. We have also shown that bFGF and VEGF induce a synergistic invasive response [47]. We investigated the effects of Trx and Trx-bp-loop on BMEC collagen gel invasion in the absence or presence of bFGF or VEGF. When added on its own, up to 1 μg/ml Trx-bp-

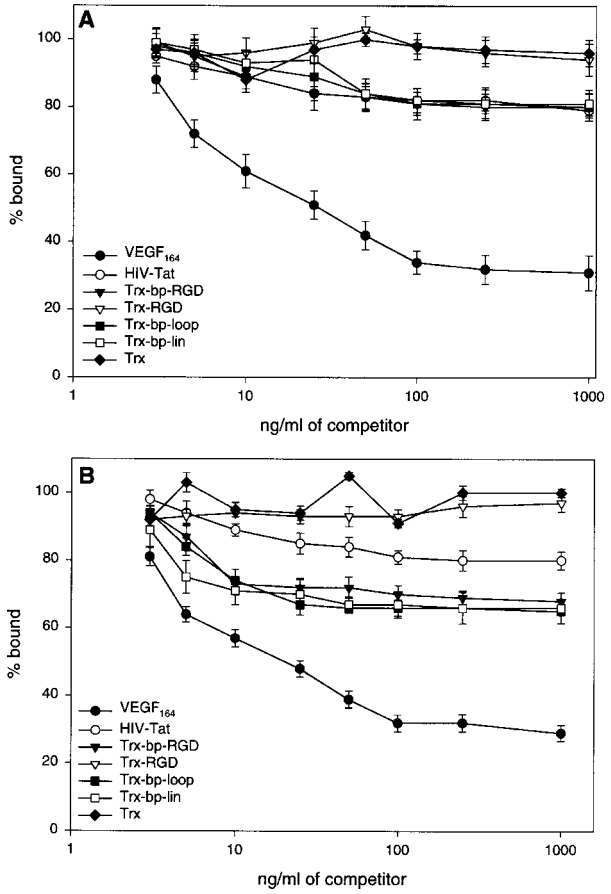

Figure 3 Competition of $VEGF_{164}$ with Trx/basic-peptide chimaeras

Shown are competition assays with radiolabelled canine $VEGF_{164}$ and Trx/basic-peptide chimaeras on PAEC-Flt-1 (**A**) and PAEC-KDR (**B**) cells. Competitor peptides used are indicated in the key.

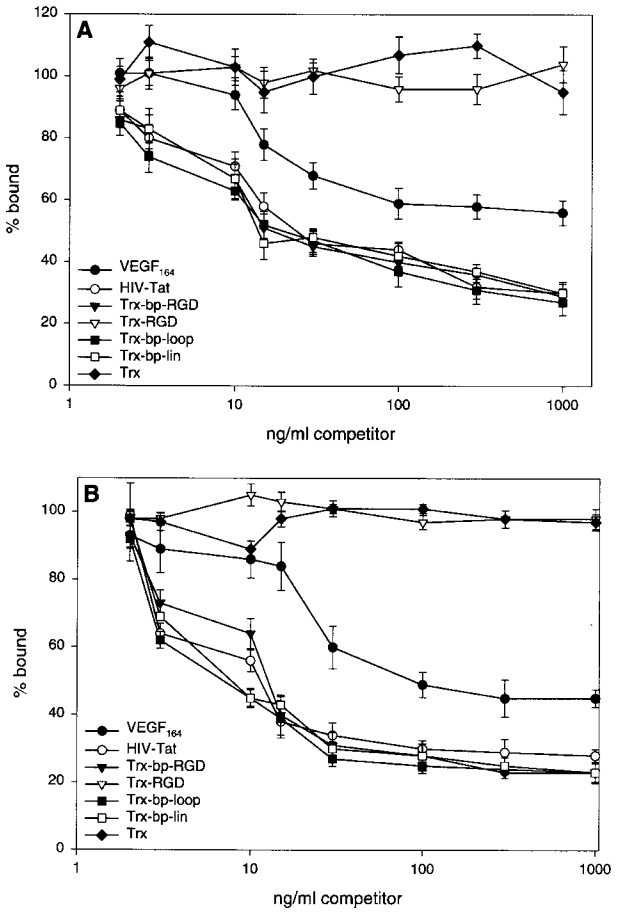

Figure 4 Competition of Trx-bp-loop with $VEGF_{164}$

Shown are competition assays with radiolabelled Trx-bp-loop on PAEC-Flt-1 (**A**) and PAEC-KDR (**B**) cells. Competitor peptides used are indicated in the key.

loop had a minimal effect. However, Trx-bp-loop potentiated the effect of bFGF in a dose-dependent manner (Figure 5), with concentrations of 300 ng/ml and 1 μg/ml reaching statistical significance ($P < 0.001$). Assuming that VEGF has an M_r of 43000 and Trx-bp-loop has an M_r of 15400, then, on an equimolar basis, 30 ng/ml VEGF would be equivalent to 10 ng/ml Trx-bp-loop. Figure 6 also shows that Trx-bp-loop was less effective than VEGF in potentiating bFGF-induced invasion. When co-added with VEGF, Trx-bp-loop appeared to reduce invasion in a dose-dependent manner (Figure 5), with a concentration of 1 μg/ml reaching statistical significance ($P < 0.02$). Unlike Trx-bp-loop, Trx had no effect under any of the conditions tested, i.e. no effect was seen in the absence or presence of bFGF or VEGF when Trx was added at concentrations up to 1 μg/ml and assessed for up to 7 days (results not shown). In addition, when the collagen gel invasion assay was performed with PAEC expressing either VEGFR-1 or -2, no tube formation was observed (results not shown).

The biological activity of Trx/basic-peptide chimaeras was also tested *in vivo* using the chorioallantoic membrane (CAM) in the chicken egg as a model system for angiogenesis. Figure 6 shows that Trx-bp-loop stimulated angiogenesis on the CAM, although to a lesser extent than VEGF. This might be due to increased turnover of this protein on the CAM, or to altered signalling through VEGFRs as compared with that induced by VEGF.

Table 2 Stimulation of endothelial cell growth with Trx/basic-peptide chimaeras

EA.hy 926 cells were grown in culture as described in the Experimental section. Cell numbers in the presence of 0.5% (v/v) serum plus the indicated peptides were determined after 72 h. Values are means ± S.E.M. for triplicate wells.

Added peptide	$10^{-3} \times$ Cell number
None	26.1 ± 2.0
Trx	26.9 ± 2.6
Trx-bp-lin	90.0 ± 2.3
Trx-bp-loop	89.6 ± 1.6
HIV-TAT	87.7 ± 3.2
$VEGF_{164}$	111.4 ± 2.3
Basic peptide	120.3 ± 1.2

Finally, we addressed the question of whether Trx/basic-peptide chimaeras and VEGF activate the same pathways in the

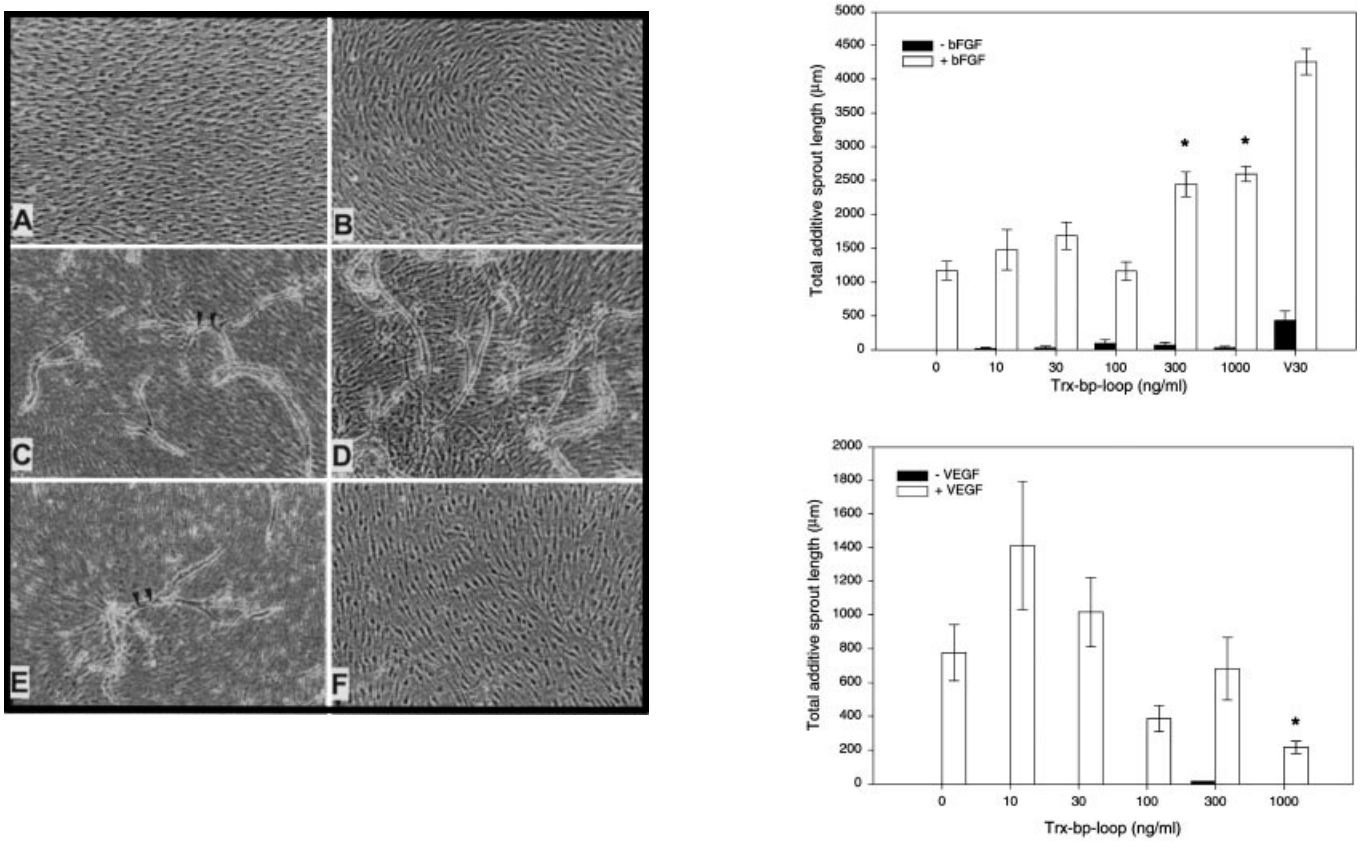

Figure 5 Collagen invasion by endothelial cells

Left panels: effect of Trx-bp-loop on collagen gel invasion by BMEC. Confluent monolayers of BMEC on three-dimensional collagen gels were treated as follows: (**A**) control; (**B**) 1 μg/ml Trx-bp-loop; (**C**) 10 ng/ml bFGF; (**D**) bFGF + Trx-bp-loop; (**E**) 30 ng/ml human recombinant VEGF; (**F**) human recombinant VEGF + Trx-bp-loop. Cultures were photographed by phase-contrast microscopy after 4 days (**A**–**D**) or 7 days (**E**, **F**). Arrowheads in (**C**) and (**E**) reveal translucent lines in BMEC cords, which are indicative of lumen formation. Magnification: $\times$ 80. Top right: Trx-bp-loop potentiates bFGF-induced collagen gel invasion by BMEC. Confluent monolayers of BMEC on three-dimensional collagen gels were treated with human recombinant VEGF (30 ng/ml; V30) or Trx-bp-loop at the indicated concentrations, in the absence or presence of bFGF (10 ng/ml). Invasion was quantified after 4 days, as described in the Experimental section. Values are means $\pm$ S.E.M. from at least four experiments per condition, i.e. at least 12 photographic fields (three fields per experiment). Significance of differences: $^*P < 0.001$. Bottom right: Trx-bp-loop inhibits VEGF-induced collagen gel invasion by BMEC. Confluent monolayers of BMEC on three-dimensional collagen gels were treated with Trx-bp-loop at the indicated concentrations, in the absence or presence of human recombinant VEGF (30 ng/ml). Invasion was quantified after 7 days, as described in the Experimental section. Values are means $\pm$ S.E.M. from at least four experiments per condition, i.e. at least 12 photographic fields (three fields per experiment). Significance of differences: $^*P < 0.02$.

cellular signalling network downstream from VEGFRs. We measured the activity of VEGFR-2 and the MAP kinase signalling cascade in PAEC and HUVEC. Figure 7(A) shows an *in vitro* kinase assay performed with HUVEC. The *in vitro* autophosphorylation activity of VEGFR-2 was stimulated by both VEGF and Trx-bp-loop, but not by a control Trx protein lacking the sequence of the TAT basic peptide. When lysates of control and agonist-stimulated PAEC were used in MAP kinase assays, we obtained the result shown in Figure 7(B). Both Trx-bp-loop and VEGF stimulated the activity of the Erk branch of the MAP kinases in PAEC expressing VEGFR-2 (left panel), whereas control PAEC lacking VEGFR-2 were not stimulated (right panel). This clearly shows that basic peptide, at least qualitatively, mimics VEGF-induced signalling via VEGFR-2. The fact that control PAEC were not activated by Trx-bp-loop supports the notion that signalling is VEGFR-specific.

DISCUSSION

Our data extend earlier studies showing that an HIV-TAT-derived peptide sequence promotes angiogenesis in a variety of systems through interaction with VEGFRs [28,33,34]. VEGFRs expressed on endothelial cells bind the basic peptide derived from HIV-TAT. This activity might explain the high incidence of Kaposi's sarcoma in AIDS patients, although additional factors, such as infection with human herpes virus 8, might contribute to these malignancies [49,50]. Our present study also suggests that TAT and VEGF apparently bind to distinct sites on both VEGFR-1 and VEGFR-2. Trx/basic-peptide chimaeras, but not Trx lacking these sequences, bound VEGFRs with high affinity. Trx-bp-loop and VEGF partially competed for binding to VEGFR-1 and -2, presumably through an allosteric effect or due to steric interference. Trx-bp-loop potently stimulated endothelial cell growth in culture. The kinase data shown in Figure 7 suggest that the underlying mechanism depends, at least in part, on MAP kinase activity. Similarly, this chimaeric protein was active in a collagen gel invasion assay; it potentiated the effect of bFGF in a manner qualitatively similar to what has been reported previously for bFGF and VEGF or VEGF-C [47,48]. However, on a molar basis, higher concentrations of Trx-bp-loop were required to observe an effect; at very high concentrations, Trx-bp-loop even inhibited the effect of VEGF. This suggests that

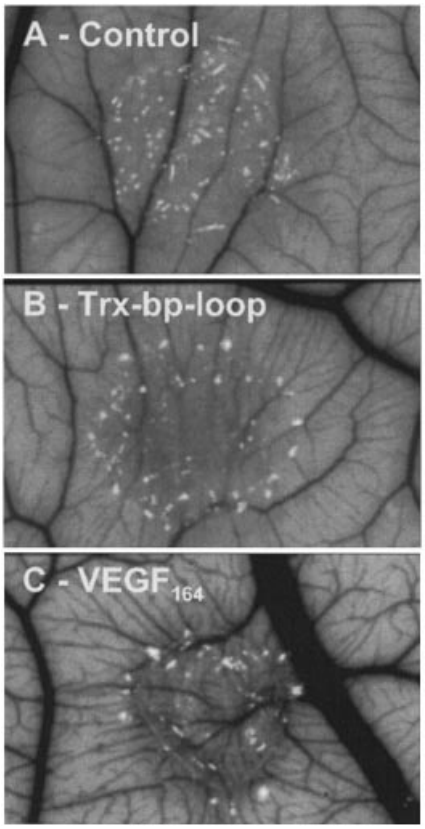

Figure 6 Induction of angiogenesis on chicken CAMs

(**A**) Control CAM; (**B**) +3 μg of Trx-bp-loop; (**C**) +3 μg of canine $VEGF_{164}$. Eggs were removed from the shell at day 3.5 after fertilization and the peptides were applied on day 8.5 on a 0.5% methyl cellulose disc. Eggs were cultivated in Petri dishes in a humidified incubator, and pictures were taken between days 9.5 and 11.

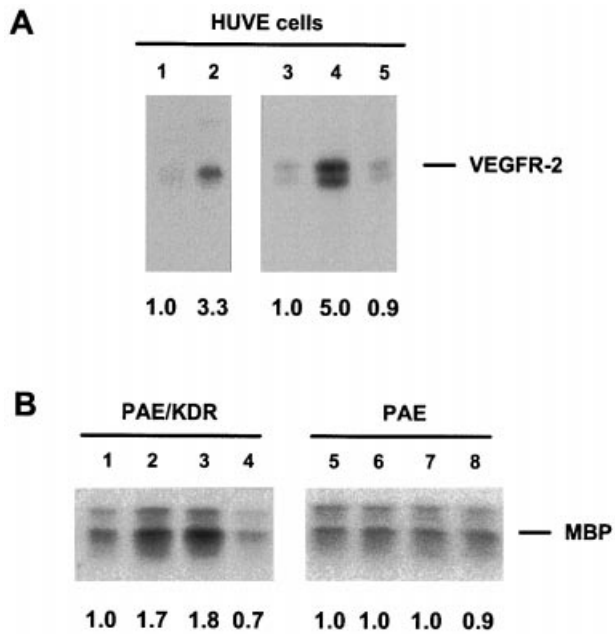

Figure 7 *In vitro* kinase activity of VEGFR-2 and MAP kinase

(**A**) Starved HUVEC were treated for 15 min with: lane 2, 2 nM canine $VEGF_{164}$; lane 4, 1 nM Trx-bp-loop; lane 5, 1 nM Trx. Lanes 1 and 3 show results from unstimulated control cells. Cells were lysed and VEGFR-2 was immunoprecipitated as described in the Experimental section. Quantification of the autoradiographs is shown below each lane; the control value was arbitrarily set to 1.0. (**B**) PAEC and PAEC-KDR cells were stimulated for 15 min with: lanes 2 and 6, 1 nM canine $VEGF_{164}$; lanes 3 and 7, Trx-bp-loop; lanes 4 and 8, 1 nM Trx. Lanes 1 and 5 show results from unstimulated control cells. Quantification of the autoradiographs is shown below each lane; the control value was arbitrarily set to 1.0. MBP, myelin basic protein.

these polypeptides compete for binding to the same receptor, yet differ somewhat in their signalling properties through VEGFRs. For instance, signalling by Trx/basic-peptide chimaeras through VEGFRs might be less efficient than with VEGF because of subtle differences in the kinetics of signalling to downstream targets. Agonistic erythropoietin mimetics isolated by peptide phage display may be precedents for such ligand behaviour [51]. These peptides evoked only a weak mitogenic response, although they bound to receptors with affinities higher than that of erythropoietin. The interpretation of those data was that proper orientation of dimerized receptor subunits is crucial for efficient receptor signalling. The different dose responses observed in the collagen invasion and CAM assays for Trx-bp-loop in our study might be similarly explained.

This leads to the interesting question of how Trx/basic-peptide chimaeras and the full-length TAT protein activate VEGFRs. Stimulation of VEGFR-2 by full-length TAT and a TAT-derived short basic peptide have been described previously, but the underlying mechanism was not elucidated [28]. Tyrosine kinase receptors undergo changes in conformation upon ligand-induced dimerization that lead to activation of the intracellular kinase domain. Several mechanisms may be envisioned for the functional interaction of the TAT peptide with VEGFRs. (i) The basic peptide and Trx/basic-peptide chimaeras might form dimers that interact with two VEGFR monomers. This is unlikely for the basic peptide, which lacks sequences required for self-association, but might play a role in signalling by Trx/basic-peptide chimaeras. (ii) Binding of the basic peptide to the extracellular domain of VEGFRs might alter the structure of monomeric receptor molecules. The acquired conformation might then be translated into structural changes in the intracellular kinase domain that unleash receptor tyrosine kinase activity. (iii) Structural changes in the extracellular receptor domain upon ligand binding might indirectly cause receptor dimerization through a structural change in receptor monomers. In all cases, receptor activation will be followed by recruitment of cellular signalling proteins into an active signal transduction complex located at the cell membrane. A detailed investigation of the mechanisms responsible for VEGFR activation by the described Trx/basic-peptide mimetics, involving biochemical and structural studies, is under way.

The fact that peptides totally unrelated to VEGF stimulate receptor activity opens interesting avenues for exploring receptor signalling, particularly since low-M_r mimetics specific for tyrosine kinase growth factor receptors have not so far been characterized in much detail. The identification of new bioactive ligands for VEGFRs also opens up new possibilities for clinical applications focusing on aberrant angiogenesis. We are currently pursuing the possibility of using these peptides as specific address tags to deliver cytotoxic reagents to tumour-induced blood vessels. Such an anti-angiogenic therapy will allow selective destruction of the tumour vasculature, thereby choking further growth of the malignant tissue, as proposed in the Introduction (reviewed in [7]). The polypeptides investigated in the present study may also

be useful for the treatment of diseases associated with decreased angiogenesis, since they behave as strong agonists for VEGFRs.

We thank Dr P. Sarmientos for bFGF, Dr M.B. Furie and Dr S.C. Silverstein for the BMEC, Dr C.-J.S. Edgell for the EA.hy 926 cells, F. Borlat (Serono Pharmaceutical Research Institute S.A., Geneva, Switzerland) for performing the endotoxin measurements, and M. Quayzin for technical assistance. We also thank Dr K. Kirschner and Dr J. Hofsteenge for critical comments on the manuscript, and Professor J. Jiricny for his continuing support for our projects. This work was supported by grants to M.S.P. from the Swiss National Science Foundation (#3100-043364.95), to K.B.-H. and R.J. from Bundesamt für Energiewirtschaft through Hauptabteilung für die Sicherheit der Kernanlagen (#63665, 65680) and to J.W. from Deutsche Forschungsgemeinschaft (#SFB451, B1). P.S. and W.W. were supported by an EU grant (#FI4PCT960043) and by Bundesamt für Bildung und Wissenschaft (#96.0123). This study was also supported in part by a grant from Deutsche Forschungsgemeinschaft (SFB451, project B1) to J.W.

REFERENCES

1 Folkman, J. and D'Amore, P. A. (1997) Blood vessel formation: What is its molecular basis? Cell **87**, 1153–1155
2 Hanahan, D. (1997) Signaling vascular morphogenesis and maintenance. Science **277**, 48–50
3 Namiki, A., Brogi, E., Kearney, M., Kim, E. A., Wu, T., Couffinhal, T., Varticovski, L. and Isner, J. M. (1995) Hypoxia induces vascular endothelial growth factor in cultured human endothelial cells. J. Biol. Chem. **270**, 31189–31195
4 Ferrara, N., Chen, H., Davis, S. T., Gerber, H. P., Nguyen, T. N., Peers, D., Chisholm, V., Hillan, K. J. and Schwall, R. H. (1998) Vascular endothelial growth factor is essential for corpus luteum angiogenesis. Nat. Med. (N. Y.) **4**, 336–340
5 Aiello, L. P. (1997) Vascular endothelial growth factor and the eye: biochemical mechanisms of action and implications for novel therapies. Ophthal. Res. **29**, 354–362
6 Sueishi, K., Yonemitsu, Y., Nakagawa, K., Kaneda, Y., Kumamoto, M. and Nakashima, Y. (1997) Atherosclerosis and angiogenesis. Its pathophysiological significance in humans as well as in an animal model induced by the gene transfer of vascular endothelial growth factor. Ann. N. Y. Acad. Sci. **811**, 311–322
7 Zetter, B. R. (1998) Angiogenesis and tumor metastasis. Annu. Rev. Med. **49**, 407–424
8 Flamme, I., Krieg, M. and Plate, K. H. (1998) Up-regulation of vascular endothelial growth factor in stromal cells of hemangioblastomas is correlated with up-regulation of the transcription factor HRF/HIF-2alpha. Am. J. Pathol. **153**, 25–29
9 Senger, D. R., Ledbetter, S. R., Claffey, K. P., Papadopoulos-Sergiou, A., Peruzzi, C. A. and Detmar, M. (1996) Stimulation of endothelial cell migration by vascular permeability factor/vascular endothelial growth factor through cooperative mechanisms involving the alphavbeta3 integrin, osteopontin, and thrombin. Am. J. Pathol. **149**, 293–305
10 Keyt, B. A., Berleau, L. T., Nguyen, H. V., Chen, H., Heinsohn, H., Vandlen, R. and Ferrara, N. (1996) The carboxyl-terminal domain (111–165) of vascular endothelial growth factor is critical for its mitogenic potency. J. Biol. Chem. **271**, 7788–7795
11 Alon, T., Hemo, I., Itin, A., Peer, J., Stone, J. and Keshet, E. (1995) Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. Nat. Med. (N. Y.) **1**, 1024–1028
12 Gerber, H. P., Dixit, V. M. and Ferrara, N. (1998) Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. J. Biol. Chem. **273**, 13313–13316
13 Gerber, H. P., McMurtrey, A., Kowalski, J., Yan, M., Keyt, B. A., Dixit, V. and Ferrara, N. (1998) Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. J. Biol. Chem. **273**, 30336–30343
14 Gupta, K., Kshirsagar, S., Li, W., Gui, L., Ramakrishnan, S., Gupta, P., Law, P. Y. and Hebbel, R. P. (1999) VEGF prevents apoptosis of human microvascular endothelial cells via opposing effects on MAPK/ERK and SAPK/JNK signaling. Exp. Cell Res. **247**, 495–504
15 Pepper, M. S., Ferrara, N., Orci, L. and Montesano, R. (1991) Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells. Biochem. Biophys. Res. Commun. **181**, 902–906
16 Wang, H. and Keiser, J. A. (1998) Vascular endothelial growth factor upregulates the expression of matrix metalloproteinases in vascular smooth muscle cells: role of flt-1. Circ. Res. **83**, 832–840
17 Roberts, W. G. and Palade, G. E. (1997) Neovasculature induced by vascular endothelial growth factor is fenestrated. Cancer Res. **57**, 765–772
18 Esser, S., Wolburg, K., Wolburg, H., Breier, G., Kurzchalia, T. and Risau, W. (1998) Vascular endothelial growth factor induces endothelial fenestrations *in vitro*. J. Cell Biol. **140**, 947–959
19 Klagsbrun, M. and D'Amore, P. A. (1996) Vascular endothelial growth factor and its receptors. Cytokine Growth Factor Rev. **7**, 259–270
20 Shibuya, M., Yamaguchi, S., Yamane, A., Ikeda, T., Tojo, A., Matsushime, H. and Sato, M. (1990) Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. Oncogene **5**, 519–524
21 Terman, B. I., Carrion, M. E., Kovacs, E., Rasmussen, B. A., Eddy, R. L. and Shows, T. B. (1991) Identification of a new endothelial cell growth factor receptor tyrosine kinase. Oncogene **6**, 1677–1683
22 Mustonen, T. and Alitalo, K. (1995) Endothelial receptor tyrosine kinases involved in angiogenesis. J. Cell Biol. **129**, 895–898
23 Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C. et al. (1996) Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature (London) **380**, 435–439
24 Ferrara, N., Carver Moore, K., Chen, H., Dowd, M., Lu, L., O Shea, K. S., Powell, B. L., Hillan, K. J. and Moore, M. W. (1996) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature (London) **380**, 439–442
25 Fong, G. H., Rossant, J., Gertsenstein, M. and Breitman, M. L. (1995) Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature (London) **376**, 66–70
26 Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X. F., Breitman, M. L. and Schuh, A. C. (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature (London) **376**, 62–66
27 Gerber, H. P., Vu, T. H., Ryan, A. M., Kowalski, J., Werb, Z. and Ferrara, N. (1999) VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat. Med. **5**, 623–628
28 Albini, A., Soldi, R., Giunciuglio, D., Giraudo, E., Benelli, R., Primo, L., Noonan, D., Salio, M., Camussi, G., Rockl, W. and Bussolino, F. (1996) The angiogenesis induced by HIV-1 tat protein is mediated by the Flk-1/KDR receptor on vascular endothelial cells. Nat. Med. (N. Y.) **2**, 1371–1375
29 Albini, A., Benelli, R., Presta, M., Rusnati, M., Ziche, M., Rubartelli, A., Paglialunga, G., Bussolino, F. and Noonan, D. (1996) HIV-tat protein is a heparin-binding angiogenic growth factor. Oncogene **12**, 289–297
30 Ensoli, B., Gendelman, R., Markham, P., Fiorelli, V., Colombini, S., Raffeld, M., Cafaro, A., Chang, H. K., Brady, J. N. and Gallo, R. C. (1994) Synergy between basic fibroblast growth factor and HIV-1 Tat protein in induction of Kaposi's sarcoma. Nature (London) **371**, 674–680
31 Albini, A., Barillari, G., Benelli, R., Gallo, R. C. and Ensoli, B. (1995) Angiogenic properties of human immunodeficiency virus type 1 Tat protein. Proc. Natl. Acad. Sci. U.S.A. **92**, 4838–4842
32 Ensoli, B., Barillari, G., Salahuddin, S. Z., Gallo, R. C. and Wong-Staal, F. (1990) Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. Nature (London) **345**, 84–86
33 Ganju, R. K., Munshi, N., Nair, B. C., Liu, Z. Y., Gill, P. and Groopman, J. E. (1998) Human immunodeficiency virus tat modulates the Flk-1/KDR receptor, mitogen-activated protein kinases and components of focal adhesion in Kaposi's sarcoma cells. J. Virol. **72**, 6131–6137
34 Mitola, S., Sozzani, S., Luini, W., Primo, L., Borsatti, A., Weich, H. and Bussolino, F. (1997) Tat-human immunodeficiency virus-1 induces human monocyte chemotaxis by activation of vascular endothelial growth factor receptor-1. Blood **90**, 1365–1372
35 Ensoli, B. and Cafaro, A. (1996) HIV-1 and Kaposi's sarcoma. Eur. J. Cancer Prevent. **5**, 410–412
36 Gallo, R. C. (1998) The enigmas of Kaposi's sarcoma. Science **282**, 1837–1839
37 Vives, E., Brodin, P. and Lebleu, B. (1997) A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. J. Biol. Chem. **272**, 16010–16017
38 Edgell, C. J., McDonald, C. C. and Graham, J. B. (1983) Permanent cell line expressing human factor VIII-related antigen established by hybridization. Proc. Natl. Acad. Sci. U.S.A. **80**, 3734–3737
39 Furie, M. B., Cramer, E. B., Naprstek, B. L. and Silverstein, S. C. (1984) Cultured endothelial cell monolayers that restrict the transendothelial passage of macromolecules and electrical current. J. Cell Biol. **98**, 1033–1041
40 Scheidegger, P., Weiglhofer, W., Suarez, S., Kaser-Hotz, B., Steiner, R., Ballmer-Hofer, K. and Jaussi, R. (1999) Vascular endothelial growth factor (VEGF) and its receptors in tumor-bearing dogs. Biol. Chem. **380**, 1449–1454
41 Montesano, R. and Orci, L. (1985) Tumor-promoting phorbol esters induce angiogenesis *in vitro*. Cell **42**, 469–477
42 Scatchard, G. (1949) The attractions of proteins for small molecules and ions. Ann. N. Y. Acad. Sci. **51**, 660–672
43 Kroll, J. and Waltenberger, J. (1997) The vascular endothelial growth factor receptor KDR activates multiple signal transduction pathways in porcine aortic endothelial cells. J. Biol. Chem. **272**, 32521–32527

44 Urich, M., El Shemerly, M. Y., Besser, D., Nagamine, Y. and Ballmer-Hofer, K. (1995) Activation and nuclear translocation of mitogen-activated protein kinases by polyomavirus middle-T or serum depend on phosphatidylinositol 3-kinase. J. Biol. Chem. **270**, 29286–29292
45 Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M. and Heldin, C. H. (1994) Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. J. Biol. Chem. **269**, 26988–26995
46 Montesano, R., Kumar, S., Orci, L. and Pepper, M. S. (1996) Synergistic effect of hyaluronan oligosaccharides and vascular endothelial growth factor on angiogenesis *in vitro*. Lab. Invest. **75**, 249–262
47 Pepper, M. S., Ferrara, N., Orci, L. and Montesano, R. (1992) Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis *in vitro*. Biochem. Biophys. Res. Commun. **189**, 824–831
48 Pepper, M. S., Mandriota, S. J., Jeltsch, M., Kumar, V. and Alitalo, K. (1998) Vascular endothelial growth factor (VEGF)-C synergizes with basic fibroblast growth factor and VEGF in the induction of angiogenesis *in vitro* and alters endothelial cell extracellular proteolytic activity. J. Cell. Physiol. **177**, 439–452
49 Boshoff, C. (1998) Coupling herpesvirus to angiogenesis. Nature (London) **391**, 24–25
50 Flore, O., Rafii, S., Ely, S., Leary, J. J., Hyjek, E. M. and Cesarman, E. (1998) Transformation of primary human endothelial cells by Kaposi's sarcoma-associated herpesvirus. Nature (London) **394**, 588–592
51 Syed, R. S., Reid, S. W., Li, C., Cheetham, J. C., Aoki, K. H., Liu, B., Zhan, H., Osslung, T. D., Chirino, A. J., Zhang, J. et al. (1998) Efficiency of signalling through cytokine receptors depends critically on receptor orientation. Nature (London) **395**, 511–516

Received 30 August 2000; accepted 2 November 2000