0026-895X/07/7106-1657–1665$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 71:1657–1665, 2007

Vol. 71, No. 6
34249/3210893
Printed in U.S.A.

Inhibition of Vascular Endothelial Growth Factor Cotranslational Translocation by the Cyclopeptolide CAM741

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Received January 19, 2007; accepted March 16, 2007

ABSTRACT

The cyclopeptolide CAM741 inhibits cotranslational translocation of vascular cell adhesion molecule 1 (VCAM1), which is dependent on its signal peptide. We now describe the identification of the signal peptide of vascular endothelial growth factor (VEGF) as the second target of CAM741. The mechanism by which the compound inhibits translocation of VEGF is very similar or identical to that of VCAM1, although the signal peptides share no obvious sequence similarities. By mutagenesis of the VEGF signal peptide, two important regions, located in the N-terminal and hydrophobic segments, were identified as critical for compound sensitivity. CAM741 alters positioning of the VEGF signal peptide at the translocon, and increasing hydrophobicity in the h-region reduces compound sensitivity and causes a different, possibly more efficient, interaction with the translocon. Although CAM741 is effective against translocation of both VEGF and VCAM1, the derivative NFI028 is able to inhibit only VCAM1, suggesting that chemical derivatization can alter not only potency, but also the specificity of the compounds.

We have recently reported that the cyclopeptolide CAM741, a derivative of the naturally occurring substance Hun-7293, inhibits cotranslational translocation of vascular cell adhesion molecule 1 (VCAM1), which is dependent on its signal peptide (SP) and occurs at the level of VCAM1 SP insertion in the Sec61 translocon (Besemer et al., 2005; Harant et al., 2006). Very similar observations were reported by Garrison et al. (2005) using cotransin, a compound with related structure. These findings demonstrate for the first time that a compound can interfere with the process of cotranslational translocation in a SP-dependent manner.

Amino-terminal, cleavable SPs, when emerging from the ribosome, are recognized by the signal recognition particle, which then directs the ribosome-nascent polypeptide chain complex to the heterotrimeric Sec61 (composed of the α-, β-, and γ subunits) complex embedded in the membrane of the endoplasmic reticulum (ER) (for review, see Rapoport et al., 1996; Hegde and Lingappa, 1997; Matlack et al., 1998; Johnson and van Waes, 1999; Stroud and Walter, 1999; Osborne et al., 2005). SPs, usually of 20 to 30 amino acid residues in length, have a typical three-domain structure, representing a frequently positively charged n-region, a hydrophobic core region (h-region), and a more polar c-region containing the site for SP cleavage (Nielsen et al., 1997; Nielsen et al., 1999). It has been assumed that all SPs interact with the translocon in a similar manner, but there is now increasing evidence that there are remarkable differences between individual SPs (reviewed by Hegde and Bernstein, 2006). Some SPs have been shown to require interaction with accessory translocon components, such as translocating chain-associated membrane protein or translocon-associated protein, whereas others do not (High et al., 1993; Mothes et al., 1994; Voigt et al., 1996; Fons et al., 2003). SPs also contain information on the timing of SP cleavage and subsequent *N*-glycosylation of the translocated chains (Rutkowski et al., 2001, 2003). During our own studies on the VCAM1 SP, we observed that mutations of the CAM741-sensitive region of the VCAM1 SP caused different association with the translocon components Sec61α and -β, indicating that these mutants are positioned differently within the translocon channel (Harant et al., 2006).

Based on our findings that the cyclopeptolide CAM741 can inhibit the process of cotranslational translocation in an SP-dependent manner, we performed a search for other sensitive

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.107.034249.

ABBREVIATIONS: VCAM1, vascular cell adhesion molecule 1; SP, signal peptide; ER, endoplasmic reticulum; VEGF, vascular endothelial growth factor-A; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; ELISA, enzyme-linked immunosorbent assay; TGF-α, transforming growth factor-α; SEAP, secreted alkaline phosphatase; HEK, human embryonic kidney; NC, nascent chain; wt, wild-type; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; BMH, bis-maleimidohexane; HUN-7293, cyclo[*N*-methyl-L-alanyl-(2*R*)-4-cyano-2-hydroxybutanoyl-(2*S*,4*R*)-2-amino-4-methyloctanoyl-*N*-methyl-L-leucyl-L-leucyl-1-methoxy-*N*-methyl-L-tryptophyl-(2*S*,4*R*)-2-amino-4-methyloctanoyl].

SPs to gain more insight into their functionality. We report here the identification of the SP of vascular endothelial growth factor-A (referred to as VEGF) as the second target of CAM741 action.

VEGF, also termed vascular permeability factor, is one of the key factors in angiogenesis and induces endothelial cell proliferation, migration, and tube formation (Keck et al., 1989; Leung et al., 1989). Apart from its essential role in neovascularization, VEGF is also involved in several pathological conditions, such as tumor angiogenesis, diabetic retinopathy, age-related macular degeneration, and psoriasis, making it an attractive target for therapeutic intervention (for review, see Cardones and Banez, 2006; Eichler et al., 2006; Roy et al., 2006). There exist six isoforms of VEGF, differing in their lengths (121, 145, 165, 183, 189, and 206 amino acid residues). These isoforms are generated by alternative mRNA splicing, differ in sequences encoded by exons 6 and 7, and are differentially expressed between different cells types (reviewed by Robinson and Stringer, 2001). However, all six VEGF isoforms are controlled by the same 26 amino acid-residue signal peptide (SP).

Materials and Methods

Materials

CAM741 and NFI028 were dissolved in dimethyl sulfoxide and stored at −20°C. Dulbecco's modified Eagle's medium (DMEM) was purchased from Invitrogen (Carlsbad, CA), and fetal calf serum (FCS) from Cambrex Bio Science Verviers S.p.r.l. (Verviers, Belgium). $VEGF_{165}$ ELISA was purchased from R&D Systems (Minneapolis, MN). Superfect was purchased from QIAGEN (Hilden, Germany). AttoPhos reagent, rabbit reticulocyte lysate, canine pancreatic microsomal membranes, and RiboMAX Large-Scale RNA production System-T7 were purchased from Promega (Madison, WI). Polyclonal Sec61α or Sec61β antisera were purchased from Millipore (Billerica, MA). Excel Gel SDS 8–18%, Excel Gel SDS 12–14% gels and Redivue [^{35}S]methionine were purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Transforming growth factor-α (TGF-α) was purchased from BD Biosciences (San Jose, CA).

Methods

ELISA. HaCaT keratinocytes were cultured in DMEM supplemented with 10% heat-inactivated FCS. Cells were seeded into 96-well plates at a density of 1.5 to 2 × 10^4 cells/well, grown to confluence, and then incubated with increasing concentrations of CAM741 for 16 h. VEGF production was stimulated by addition of 50 ng/ml TGF-α for 24 h. Supernatants were collected and analyzed for $VEGF_{165}$ by ELISA.

Plasmid Constructions. The SP-secreted alkaline phosphatase (SEAP) fusion constructs, VEGF SP mutants fused to the SEAP mature domain, the construct encoding a fusion of the N-terminal tag to the VEGF SP, and the truncated VEGF cDNAs encoding either 81 or 131 amino acid residues were generated by polymerase chain reaction and subcloned into pcDNA3.1 (Invitrogen). All constructs were confirmed by sequencing. The numbering of amino acid residues refers to VEGF plus SP.

Truncated cDNAs lacking a stop codon were generated by restriction digestion of the respective plasmid DNAs. In case of the SEAP fusion constructs, plasmids were linearized by digestion with either BamHI (encoding 54 amino acid residues, SEAP mature domain) or BstEII (encoding 146 amino acid residues, SEAP mature domain). Linearized plasmids were used as templates for creation of RNAs by the RiboMAX Large Scale RNA production System-T7.

Transient Transfections of HEK293 Cells. HEK293 cells were cultivated in DMEM supplemented with 10% FCS and passaged twice a week. For transfection, 1.5 × 10^4 cells in a volume of 100 μl were seeded in each well of a 96-well plate, transfected with 0.2 μg of plasmid DNA and 0.5 μl of Superfect in each well and treated with increasing concentrations of CAM741 or NFI028. Supernatants were harvested after 24 h, and analyzed for SEAP secretion using the AttoPhos reagent. Fluorescence was recorded using the SPEKTRAmax GEMINI XS (Molecular Devices, Sunnyvale, CA).

In Vitro Translocation Experiments. In vitro translation, targeting and translocation assays, and chemical cross-linking were performed with truncated RNAs using rabbit reticulocyte lysate, canine pancreatic microsomal membranes (Promega), and [^{35}S]methionine (GE Healthcare), in the presence of CAM741 or dimethyl sulfoxide (vehicle control) as described previously (Besemer et al., 2005; Harant et al., 2006). Immunoprecipitations were performed with a polyclonal Sec61α or Sec61β antiserum. Proteins were separated on Excel Gel SDS 8–18% or, where stated, on high resolution Excel Gel SDS 12–14% gels. Fixed and dried gels were exposed to X-ray films.

Results

CAM741 Inhibited Cotranslational Translocation of VEGF, Which Was Dependent on Its SP. To search for SPs, which, apart from the known VCAM1 SP (Besemer et al., 2005), could be sensitive to inhibition of translocation by CAM741, a panel of 10 different SPs fused to the mature region of secreted alkaline phosphatase (SEAP) was tested as described in Table 1. The SPs were selected as representatives of different classes of secreted or membrane proteins involved in inflammation, immune regulation and angiogenesis. The chemokines CCL22 (macrophage-derived chemokine), CCL2 (monocyte chemoattractant protein), and

TABLE 1
Different sensitivity of SP-SEAP fusion constructs to inhibition by CAM741
HEK293 cells were transfected with different SP-SEAP fusion constructs (in some cases SP + additional residues of the mature domain) and incubated with increasing concentrations of CAM741. Twenty-four hours after transfection, supernatants were harvested and analyzed for alkaline phosphatase activity. Results shown are IC_{50} values from at least three independent experiments performed in triplicates. The cleavage site is underlined; amino acid residues of the mature region are bold.

Signal peptide	Sequence	CAM741 IC_{50}	Inhibition at 10 μM
		nM	
CCL22 + 10	MARLQTALLVVLVLLAVALQATEA-**GPYGANMEDS**	>10,000	
CCL2 + 10	MKVSAALLCLLLIAATFIPQGLA-**QPDAINAPVT**	>10,000	23%
IL-13 SP + 10	MALLLTTVIALTCLGGFASP-**GPVPPSTALR**	>10,000	27%
IFN-γ + 5	MKYTSYILAFQLCIVLGSLGCYC-**QDPYV**	>10,000	45%
CCR7 SP + 10	MDLGKPMKSVLVVALLVIFQVCLC-**QDEVTDDYIG**	>10,000	49%
E-selectin	MIASQFLSALTLVLLIKESGA-	9753 ± 1791	
IL-12p40 + 3	MCHQQLVISWFSLVFLASPLVA-**IWE**	4313 ± 1381	
CXCL8 + 7	MTSKLAVALLAAFLISAALC-**EGAVLPR**	1473 ± 764	
ICAM-1 + 5	MAPSSPRPALPALLVLLGALFPGPGNA-**QTSVS**	687 ± 220	
VEGF	MNFLLSWVHWSLALLLYLHHAKWSQA-	125 ± 82	

IL, interleukin; IFN, interferon.

CXCL8 (interleukin-8) were included. The inflammatory cytokines interferon-γ, interleukin-12p40 subunit, and interleukin-13 were also used, as was VEGF. From the membrane proteins, the SP of the chemokine C-C motif receptor 7, E-selectin, and intercellular adhesion molecule-1 were chosen. Although seven of the SP-SEAP constructs showed only partial inhibition of SEAP release at the highest concentration of CAM741 (10 μM), SEAP fusion constructs of CXCL8 and the ICAM1 SPs showed some sensitivity to inhibition by CAM741 (Table 1). However, of all SPs tested, that of VEGF was identified as most sensitive to inhibition by CAM741, being only 4-fold less sensitive than the VCAM1 SP (Harant et al., 2006) (Table 1).

To determine that inhibition of SEAP release of the transfected VEGF SP fusion construct by CAM741 also occurs at the level of cotranslational translocation, truncated RNAs encoding the VEGF SP fused to 146 amino acid residues of the SEAP mature domain were used for in vitro translocation experiments. The SEAP mature domain contains a glycosylation site at position 122, and glycosylation and protection from exogenous proteases therefore indicate translocation to the ER lumen after release of the nascent chains (NCs) from the ribosome by high salt/puromycin. In vitro translocation of truncated VEGF SP-SEAP NCs produced two glycosylated fragments, which were protected from protease digestion (Fig. 1A, left). Glycosylation was confirmed by treatment with endoglycosidase F (Fig. 1A, right). The slower migrating glycosylated fragment represents the VEGF SP-SEAP construct with the SP still attached to it, whereas the faster migrating glycosylated fragment represents the SEAP mature region after SP cleavage. The fastest migrating band, representing unprocessed NCs, was almost completely degraded by proteinase K treatment (Fig. 1A, left). However, in the presence of 1 μM CAM741, formation of both glycosylated fragments was inhibited, and again the remaining, unprocessed fragment was degraded by added protease (Fig. 1A, left).

To exclude any effect of the SEAP fusion partner on the VEGF SP, we also generated 131 amino acid residue translocation intermediates from wild-type (wt) $VEGF_{165}$, which contains an *N*-glycosylation site at position 101 (position 75 of mature VEGF) (Fig. 1B, middle). In the control reaction, a slower migrating, glycosylated fragment was formed only in the presence of microsomal membranes, which was resistant to degradation by proteinase K. This fragment could be degraded only after permeabilization of the membranes with Triton X-100, whereas the residual unglycosylated product was almost completely degraded also in the absence of detergent. However, in the presence of CAM741, only the unglycosylated fragment formed which was largely degraded by proteinase K (Fig. 1B, right). Although CAM741 does not prevent formation of the tight, salt-resistant binding of VEGF NCs to the translocon, their translocation to the ER is prevented by the compound, witnessed by lack of SP cleavage and glycosylation and their sensitivity to degradation by exogenous protease.

Finally, the effect of CAM741 on release of endogenously expressed VEGF was analyzed. HaCaT keratinocytes upregulate the splice variant $VEGF_{165}$ in response to treatment with TGF-α (Gille et al., 1998). However, preincubation of HaCaT cells with increasing concentrations of CAM741 dose dependently inhibited TGF-α-induced $VEGF_{165}$ release, as determined by ELISA (Fig. 2).

Mapping of the CAM741-Sensitive Region of the VEGF SP. Although the VEGF SP is sensitive to translocation inhibition by CAM741, it shares no similarities with the highly sensitive VCAM1 SP within the primary amino acid sequence apart from an identical cleavage site for the signal peptidase complex (Table 3). However, the cleavage site of the VCAM1 SP could be replaced without loss in sensitivity, indicating that it does not contain key residues required for the compound effect (Harant et al., 2006). To identify the region critical for inhibition by CAM741, mutagenesis of the VEGF SP was performed. Mutations at different positions of

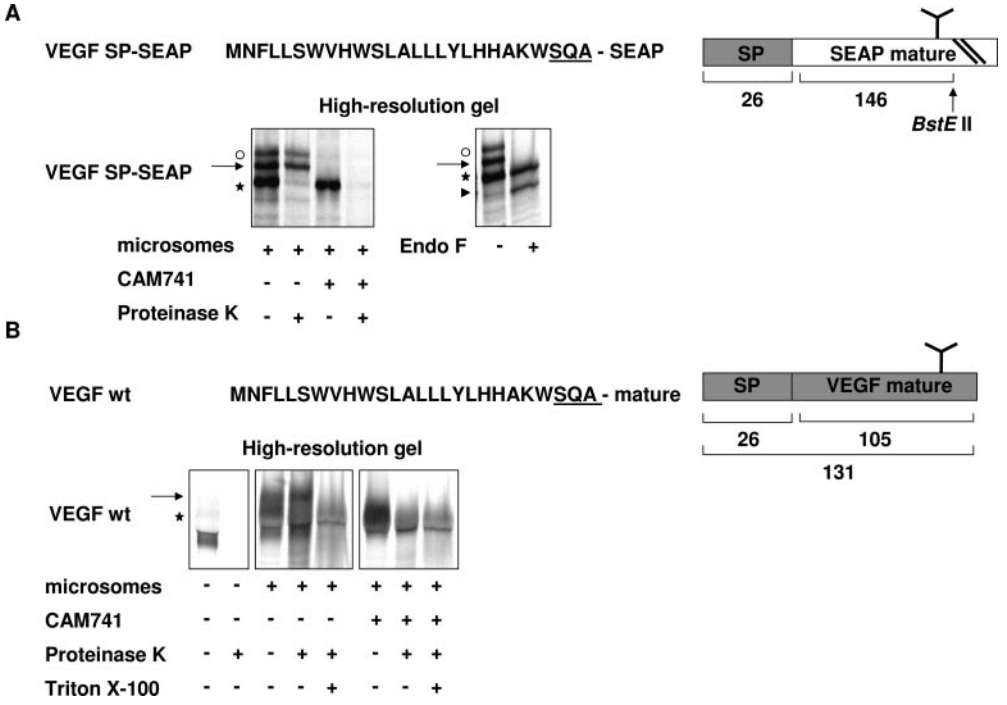

Fig. 1. CAM741 inhibits cotranslational translocation of VEGF SP-SEAP. A, sequence and schematic representation of the construct used. In vitro translocation of truncated VEGF SP-SEAP NCs (SP + 146 amino acid residues SEAP mature domain) in the absence or presence of CAM741 (1 μM), either untreated or treated with proteinase K (left); deglycosylation of sedimented VEGF SP-SEAP NCs with endoglycosidase F (Endo F; right). B, schematic representation of the construct used. In vitro translocation of truncated 131 amino acid residues VEGF NCs in the absence of microsomes (left), in the presence of microsomes (middle), or in the presence of microsomes and 1 μM CAM741 (right), either untreated or treated with proteinase K, or proteinase K and 1% Triton X-100. ○, glycosylated NCs with the SP attached; →, glycosylated NCs without SP; ⋆, nonprocessed NCs; ▸, NCs with SP cleaved off.

the VEGF SP caused changes in compound sensitivity; two regions, the n-region and parts of the h-region, were recognized as being essential for compound sensitivity (Table 2). The contribution of the n-region to compound sensitivity was identified by removal of the N-terminal amino acid residues 2 to 5 or by changing leucines at positions 4 and 5 into glutamine residues, because both mutants required higher concentrations of CAM741 for inhibition (Table 2). However, although these results strongly indicate an involvement of the n-region in compound sensitivity, it is dispensable for successful translocation, in that SEAP secretion was not affected in these less sensitive mutants.

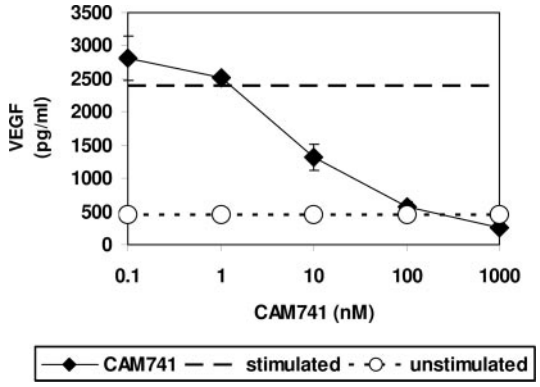

Fig. 2. CAM741 inhibits release of endogenously expressed $VEGF_{165}$. HaCaT cells were treated with TGF-α in the presence of increasing concentrations of CAM741. The concentration of released $VEGF_{165}$ (picograms per milliliter) was determined by ELISA.

Two residues within the central hydrophobic h-region were identified as critical for CAM741 sensitivity: leucine 12 and alanine 13. Substitutions of these residues by different aliphatic residues generated VEGF SP mutants with either increased or decreased compound sensitivities. Replacing leucine 12 with valine resulted in a mild decrease in sensitivity, whereas a more pronounced decrease was observed with isoleucine at this position. When alanine 13 was when changed to valine, leucine, or isoleucine, again a slight decrease was observed for valine, but a clear reduction in sensitivity for leucine or isoleucine at this position. However, when changing Leu12 and Ala13 to valines or isoleucines, sensitivity to CAM741 was further decreased (Table 2).

Conversely, reducing hydrophobicity at these positions by replacing leucine 12 with glycine caused enhancement in sensitivity, as did the conversion into alanine. However, substitution of both Leu12 and Ala13 with glycine residues resulted in a hypersensitive VEGF SP variant that was inhibited by CAM741 at low nanomolar concentrations (Table 2).

TABLE 2

Modulation of the sensitivity to CAM741 by mutations in the VEGF SP

HEK293 cells were transfected with different VEGF SP-SEAP fusion constructs and incubated with increasing concentrations of CAM741. Twenty-four hours after transfection, supernatants were harvested and analyzed for alkaline phosphatase activity. Results shown are IC_{50} values from at least three independent experiments performed in triplicates. Mutations are indicated by double underlining, the cleavage site is single-underlined, and amino-acid residues of the SEAP mature region are italic.

VEGF Signal Peptide	Sequence	CAM741 IC_{50}
		nM
	n-region **h-region** *c-region*	
	MNFLLSWVHW**SLALLLYL***HHAKWSQA*	
n-region		
wt	MNFLLSWVHWSLALLLYLHHAKWSQA	125 ± 82
Δ 2–5	MSWVHWSLALLLYLHHAKWSQA	1247 ± 345
L4G, L5G	MNFGGSWVHWSLALLLYLHHAKWSQA	312 ± 108
L4Q, L5Q	MNFQQSWVHWSLALLLYLHHAKWSQA	1007 ± 408
h-region		
W7H	MNFLLSHVHWSLALLLYLHHAKWSQA	58 ± 38
H9F	MNFLLSWVFWSLALLLYLHHAKWSQA	93 ± 76
W10G	MNFLLSWVHGSLALLLYLHHAKWSQA	133 ± 89
L12G	MNFLLSWVHWSGALLLYLHHAKWSQA	36 ± 21
A13G	MNFLLSWVHWSLGLLLYLHHAKWSQA	82 ± 53
L12G, A13G	MNFLLSWVHWSGGLLLYLHHAKWSQA	1.7 ± 1.0
L12A	MNFLLSWVHWSAALLLYLHHAKWSQA	32 ± 8
L12V	MNFLLSWVHWSVALLLYLHHAKWSQA	295 ± 110
A13V	MNFLLSWVHWSLVLLLYLHHAKWSQA	340 ± 103
L12V, A13V	MNFLLSWVHWSVVLLLYLHHAKWSQA	1148 ± 559
A13L	MNFLLSWVHWSLLLLLYLHHAKWSQA	2670 ± 757
L12I	MNFLLSWVHWSIALLLYLHHAKWSQA	883 ± 297
A13I	MNFLLSWVHWSLILLLYLHHAKWSQA	1484 ± 220
L12I, A13I	MNFLLSWVHWSIILLLYLHHAKWSQA	2133 ± 741
A13P	MNFLLSWVHWSLPLLLYLHHAKWSQA	6.5 ± 3.1
L14A, L15A	MNFLLSWVHWSLAAALYLHHAKWSQA	56 ± 18
L14G, L15G	MNFLLSWVHWSLAGGLYLHHAKWSQA	145 ± 69
L16V, L18V	MNFLLSWVHWSLALLVYVHHAKWSQA	648 ± 204
Y17P	MNFLLSWVHWSLALLLPLHHAKWSQA	12 ± 11
Δ 2–5 Y17P	MSWVHWSLALLLPLHHAKWSQA	312 ± 162
c-region		
H19A	MNFLLSWVHWSLALLLYLAHAKWSQA	580 ± 211
H20A	MNFLLSWVHWSLALLLYLHAAKWSQA	766 ± 295
H19A, H20A	MNFLLSWVHWSLALLLYLAAAKWSQA	473 ± 162
K22E	MNFLLSWVHWSLALLLYLHHAEWSQA	378 ± 176
W23V	MNFLLSWVHWSLALLLYLHHAKVSQA	475 ± 188
V30C	MNFLLSWVHWSLALLLYLHHAKWSQA-*IIPC*	177 ± 133
Δ 2–5, V30C	MSWVHWSLALLLYLHHAKWSQA-*IIPC*	3803 ± 476
L12G, A13G, V30C	MNFLLSWVHWSGGLLLYLHHAKWSQA-*IIPC*	7.7 ± 5.8
L12I, A13I, V30C	MNFLLSWVHWSIILLLYLHHAKWSQA-*IIPC*	4707 ± 1219

Taken together, these data indicate that the sensitivity to CAM741 can be modulated by increasing or decreasing hydrophobicity and/or size of the aliphatic residues at positions 12 and 13.

Changing of other residues in the h-region, such as leucines 14 and 15, to either alanines or glycines did not affect sensitivity to CAM741. However, conversion of leucines at position 16 and 18 into valines also caused reduction in sensitivity (Table 2).

Apart from hydrophobicity, a specific presentation of residues of the h-region may be required for the inhibitory effect of CAM741. Proline, a residue with known helix-breaking potential, was introduced at two different positions within the h-region. When alanine 13 was converted to proline, sensitivity to CAM741 markedly increased. In addition, changing tyrosine at position 17, a polar residue between leucines 16 and 18, into proline (Y17P) clearly increased sensitivity to CAM741. The results from both mutants would suggest that a specific optimal conformational presentation of residues of the h-region mediates sensitivity to CAM741, which additionally involves the n-region. We therefore tested the effect of removal of the N-terminal residues 2 to 5 in the highly sensitive VEGF SP mutant Y17P and show that in the absence of these residues, sensitivity was clearly reduced (Table 2). These data show that both the n- and h-regions mediate compound sensitivity, and maximal inhibition by CAM741 requires an interplay between these two segments.

The response of selected VEGF SP mutants to inhibition by CAM741 at the level of cotranslational translocation was analyzed by in vitro translocation assays of truncated VEGF SP-SEAP NCs (all fused to 146 amino acid residues SEAP mature domain) in the presence of increasing concentrations of CAM741. The results from the transient transfections were also reflected by these experiments (Fig. 3).

Altered Orientation of the VEGF NCs Relative to the Translocon Component Sec61β by CAM741. Targeting of the VCAM1 NCs to the translocon is not prevented by CAM741, but translocation inhibition occurs at the step of VCAM1 SP insertion into the translocon (Besemer et al., 2005; Harant et al., 2006). As the VEGF NCs could also be sedimented with the microsomal membranes after high salt/puromycin treatment in the presence of CAM741, we analyzed targeted VEGF NCs at the translocon by chemical cross-linking experiments.

When using the heterobifunctional cross-linker *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS), targeted VCAM1 NCs can be cross-linked to Sec61α in both the absence and the presence of CAM741. However, chemical cross-linking with the homobifunctional cysteine-reactive cross-linker bis-maleimidohexane (BMH) was observed only in the presence of compound a clearly enhanced cross-link to Sec61β, suggesting an altered orientation of the VCAM1 NCs toward this translocon component (Besemer et al., 2005). This observation was also shared with Garrison et al. (2005) using the structurally related compound cotransin. We now employed the same chemical cross-linkers to study the translocon environment of targeted 81 amino acid residues VEGF NCs. When subjected to cross-linking with MBS followed by immunoprecipitation with a Sec61α antiserum, cross-links to Sec61α were observed in both the absence and the presence of CAM741 (Fig. 4A, left, labeled MBS). Chemical cross-linking was then performed with BMH followed by immunoprecipitation with a Sec61β antiserum. When using this cross-linker, visible cross-links can only form between the cysteine residues present in the mature region of VEGF (positions 52 and 77), and the single cysteine residue in the cytosolic tail of Sec61β. However, only cysteine at position 52 is likely to be accessible to cross-linker, because cysteine at position 77 is too close to the peptidyl transferase site. Although cysteine 52 may also still be located within the ribosome, its distance from the peptidyl transferase site is sufficient for chemical cross-linking to the cytoplasmic tail of Sec61β, as reported for opsin NCs (Laird and High, 1997). In the presence of CAM741, an enhanced cross-link between the VEGF NCs and Sec61β was observed, although basal cross-links to Sec61β were already seen in the absence of compound, and their enhancement by CAM741 was less pronounced (Fig. 4A, right, labeled BMH) compared with targeted VCAM1 NCs (Besemer et al., 2005). The identity of other cross-linked products that formed differently between control and CAM741-treated reactions is currently unknown (Fig. 4A, open arrowheads), with similarly sized products

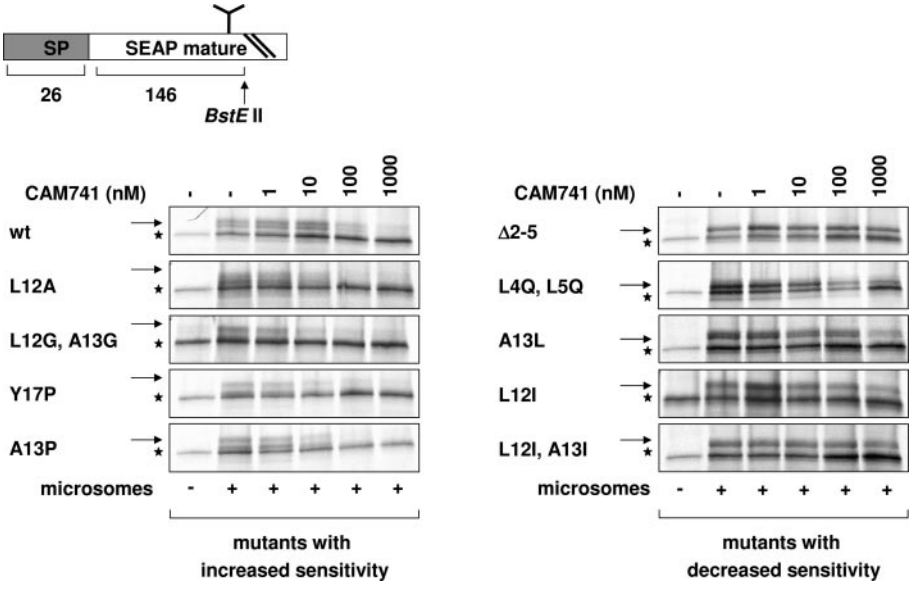

Fig. 3. Differential sensitivity of VEGF SP mutants to inhibition by CAM741. Schematic representation of the constructs used. In vitro translocation of fusion constructs of VEGF SP mutants and the 146 amino acid residues SEAP mature domain in the absence of microsomal membranes or in the presence of microsomes and increasing concentrations of CAM741. →, glycosylated NCs; ⋆, unprocessed NCs.

also formed with targeted VCAM1 NCs (Besemer et al., 2005).

CAM741 Differentially Altered Positioning of VEGF SP Mutants at the Translocon. In the presence of CAM741, not only the VCAM1 mature region but also the VCAM1 SP has an altered orientation relative to Sec61β (Harant et al., 2006). We asked whether this is also true for VEGF. Valine 30 (fourth amino acid residue of the SEAP mature domain) was replaced by a cysteine residue in the wt VEGF SP-SEAP construct, the highly sensitive mutant VEGF (L12G, A13G) SP-SEAP, and in the constructs VEGF (L12I, A13I) SP-SEAP and VEGF (Δ2–5) SP-SEAP, which have greatly reduced sensitivities to inhibition by CAM741. We chose position 30 for this substitution to exclude any effect of potential SP cleavage on cross-linking of targeted NCs, because it is located downstream of the cleavage site but near the SP. The constructs were then evaluated by transient transfections of HEK293 cells. As shown in Table 2, the constructs displayed a pattern of CAM741 sensitivity comparable with those containing valine at position 30, although overall sensitivity of the mutants was lower with cysteine at this position.

Short translocation intermediates (VEGF SP + 54 amino acid residues SEAP mature domain) were generated and targeted nascent chains subjected to chemical cross-linking with BMH and immunoprecipitation with a Sec61β antiserum. At this length of the NCs, the only cysteine residue available for cross-linking was provided at position 30 (Fig. 4B). Similar to the VCAM1 SP (Harant et al., 2006), a dose-dependent increase in Sec61β cross-links of targeted VEGF (V30C) SP-SEAP NCs by CAM741 was observed. However, as also observed for wt VEGF NCs of 81 amino acid residues (Fig. 4A), some basal cross-links to Sec61β were already detected in the absence of compound (Fig. 4B). The highly sensitive construct VEGF (L12G, A13G, V30C) SP-SEAP showed only weak cross-links to Sec61β in the vehicle-treated reaction, but also formation of Sec61β cross-links with increasing concentrations of CAM741. In contrast, the construct VEGF (L12I, A13I, V30C) SP-SEAP with greatly reduced sensitivity to CAM741, formed basal cross-links to Sec61β in the absence of compound that were not enhanced by the presence of CAM741 (Fig. 4B). However, the VEGF SP mutant lacking the amino acid residues 2 to 5 [VEGF (Δ2–5, V30C) SP-SEAP], although having strongly reduced sensitivity to CAM741, showed only weak basal cross-links with Sec61β and some enhanced formation of the Sec61β cross-link only at 1 μM CAM741 (Fig. 4B). These data indicate that substitution of Leu12 and Ala13 with isoleucine affects SP association with the translocon and could indicate a different and more efficient interaction with the translocon. In contrast, the presence of the glycines at positions 12 and 13 could cause inefficient association of the SP with the translocon, suggesting that the amino acid residues within the h-region may control the strength of translocon binding. The construct VEGF (Δ2–5) SP-SEAP, which lacked the N-terminal amino acid residues 2 to 5 but contained no mutations within the h-region, showed only weak basal cross-links to Sec61β, which further supports the idea that basal cross-linking to Sec61β was mediated through the h-region. However, the individual sensitivities of the VEGF SP mutants to CAM741 were mirrored by the different concentrations of compound required to induce Sec61β cross-links.

CAM741 Inhibited N-Terminal Translocation of a Tag Fused to the VEGF SP. Translocation of a 17-amino acid residue tag (N-tag) fused to the VCAM1 SP was inhibited by CAM741, which additionally indicated incorrect SP insertion into the translocon channel caused by the presence of compound (Harant et al., 2006). We therefore performed targeting of 81 amino acid residue VEGF NCs fused to the 17 residues N-tag in the presence or absence of CAM741. This tag contains a diagnostic glycosylation site according to the constructs used by Heinrich et al. (2000), and N-terminal translocation of the tag is visualized by glycosylation (Fig. 5). Glycosylation can only occur at the N-tag, as at a chain length of 81 amino acid residues, the wt VEGF does not contain any glycosylation sites (Fig. 5, top left). Glycosylation of the N-terminal tag was further confirmed by treatment with endoglycosidase F (Fig. 5, top right). However, in the presence of the N-tag, efficient glycosylation was seen in the control reaction but was clearly reduced when CAM741 was present (Fig. 5, top panel). In addition, inhibition of N-terminal translocation of the tag by CAM741 was dose-dependent (Fig. 5, bottom panel). Together, these data indicate altered insertion of the VEGF SP in the presence of compound.

Differential Sensitivity of the VEGF and VCAM1 SPs to the Cyclopeptolide Derivative NFI028. In addition to side chain modification of HUN-7293, providing compounds such as CAM741, variation of the peptidic

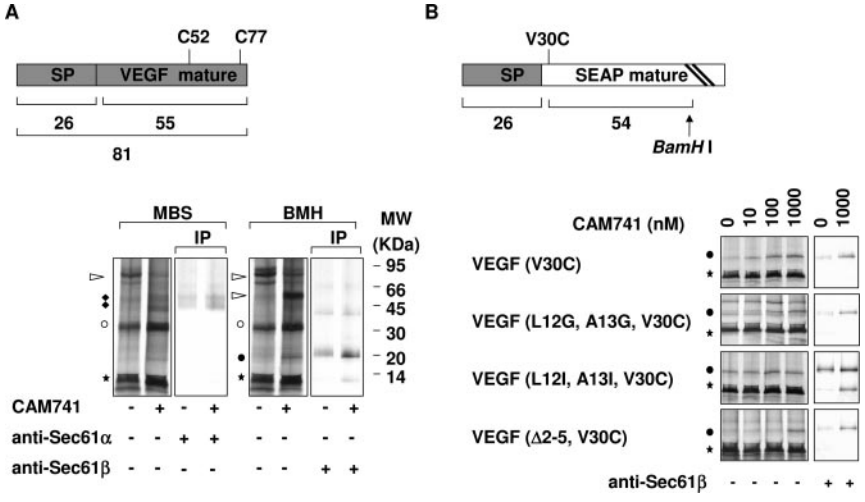

Fig. 4. CAM741 alters positioning of the VEGF NCs at the translocon. A, schematic representation of the construct used. In vitro targeting and chemical cross-linking with either MBS (left, labeled MBS) or BMH (right, labeled BMH) of truncated 81 amino acid residues VEGF NCs and immunoprecipitation with a Sec61α or Sec61β antiserum in the absence or presence of CAM741 (1 μM). ◆, Sec61α cross-link; ●, Sec61β cross-link; ⋆, nonprocessed NCs; ▷, additional unspecified cross-links; ○, residual peptidyl-tRNA-NCs. B, schematic representation of the constructs used. In vitro targeting and chemical cross-linking with BMH of truncated VEGF SP mutants fused to 54 amino acid residues SEAP mature domain containing a cysteine at position 30 (V30C; fourth position of the SEAP mature domain; left) and immunoprecipitation with a Sec61β antiserum (right). ●, Sec61β cross-link; ⋆, NCs.

backbone was investigated. These efforts disclosed cyclic hexapeptides such as NFI028 as novel structural type of potent inhibitor of VCAM-1 expression (Schreiner et al., 2003; Fig. 6). To determine whether this fundamental structural difference has an impact on VCAM1 and VEGF SP-dependent translocation, HEK293 cells were transiently transfected with VCAM1 (Δ2–10) SP-SEAP or VEGF SP-SEAP, and treated with increasing concentrations of NFI028. Although this compound was able to inhibit SEAP release from cells transfected with VCAM1 (Δ2–10) SP-SEAP at low concentrations, it did not inhibit release of SEAP by cells transfected with VEGF SP-SEAP, demonstrating that although both SPs respond to CAM741, the VEGF SP shows no sensitivity to this derivative (Table 3). Based on the observations above, that the sensitivity of the VEGF SP to CAM741 could be enhanced by mutations within the h-region, we tested whether three of the highly sensitive mutants, VEGF (A13P) SP-SEAP, VEGF (Y17P) SP-SEAP, and VEGF (L12G, A13G) SP-SEAP could respond to inhibition by NFI028. However, only a partial response to NFI028 was observed with the mutants VEGF (A13P) SP-SEAP or VEGF (Y17P) SP-SEAP. The highly CAM741-sensitive mutant VEGF (L12G, A13G) SP-SEAP showed some increased sensitivity to NFI028, although it was markedly reduced compared with CAM741 (Table 3). These data indicate that, apart from general similarities between the VEGF and VCAM1 SPs that make them susceptible to inhibition by CAM741, differences in the composition of the SP may account for NFI028 selectivity.

Discussion

Inhibition of cotranslational translocation through the SP was identified by us as a novel approach to interfere with the expression of proteins undergoing the secretory pathway. The proof of concept was provided by the discovery of the cyclopeptolide CAM741, which potently inhibits cotranslational translocation of VCAM1 (Besemer et al., 2005). At the same time, cotransin, a compound of similar structure and activity has been reported by Garrison et al. (2005). The mechanism by which this process is inhibited has been shown to be dependent on the VCAM1 SP at the level of its attachment to the Sec61 translocon (Besemer et al., 2005; Garrison et al., 2005; Harant et al., 2006). We were interested whether other SPs would also be able to respond to CAM741 to learn more about this mechanism. Garrison et al. reported some SPs with partial sensitivity to cotransin, which however lack any obvious consensus motif in their sequences (Garrison et al., 2005).

From a panel of SPs analyzed, the most sensitive SP identified was that of VEGF; inhibition of VEGF SP-SEAP release required only 4-fold higher concentrations of CAM741 than inhibition of VCAM1 SP-SEAP (Besemer et al., 2005; Harant et al., 2006). By in vitro translocation experiments, we provide evidence that this inhibition also occurs at the level of cotranslational translocation. Moreover, the mechanism seems to be very similar or identical to that observed for VCAM1 translocation inhibition, although the two SPs share no obvious similarities within their primary sequences.

These findings prompted us to analyze the features of

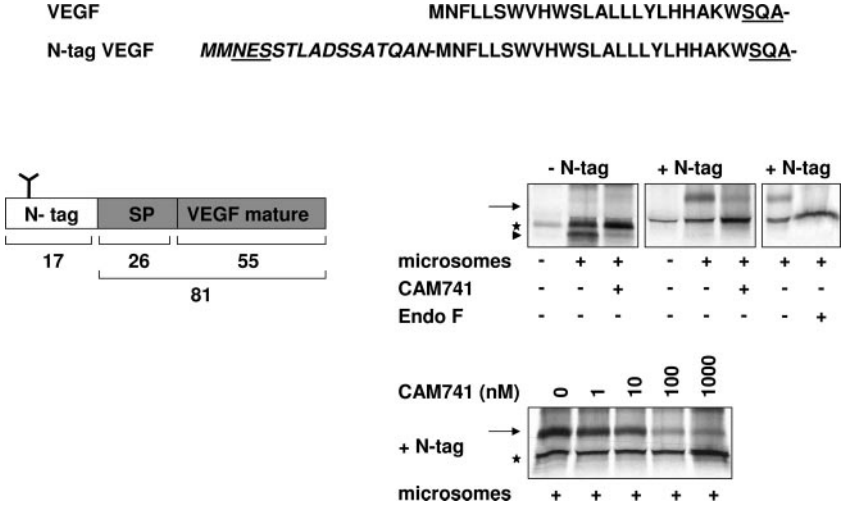

Fig. 5. CAM741 inhibits N-terminal translocation of a 17 amino acid residue tag fused to the VEGF SP. Sequences and schematic representation of the constructs. The N-terminal tag is indicated in italic letters, the glycosylation site is underlined. In vitro targeting of truncated 81 amino acid residue VEGF NCs (top left), or 81 amino acid residues VEGF NCs containing the 17 amino acid residue N-terminal tag in the absence of microsomes, or in the presence of microsomes with or without 1 μM CAM741 (top middle). Deglycosylation with endoglycosidase F (Endo F; top right). In vitro targeting of 81 amino acid residues VEGF NCs containing the 17 amino acid residues N-terminal tag in the presence of increasing concentrations of CAM741 (bottom). →, glycosylated NCs; ⋆, unprocessed NCs; ▸, NCs with SP cleaved off.

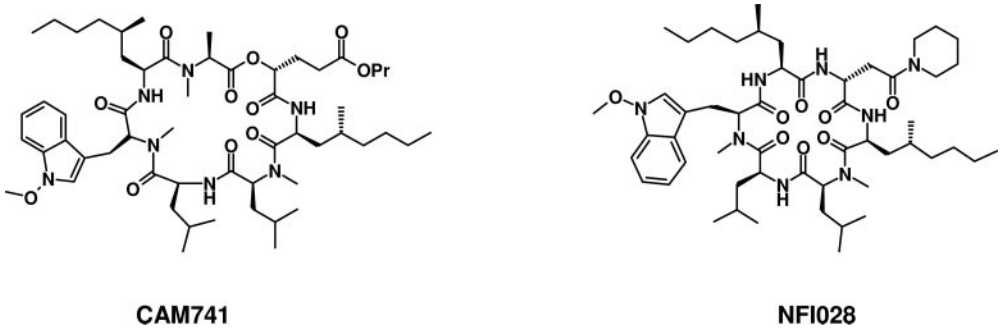

Fig. 6. Structures of CAM741 and NFI028

the VEGF SP responsible for inhibition. In contrast to the VCAM1 SP, where the critical residues are located in the h-region and the polar c-region upstream of the cleavage site (Harant et al., 2006), residues of the VEGF SP required for translocation inhibition are located in the n-region and the h-region, where positions Leu12 and Ala13 were identified as most critical for sensitivity. Aliphatic residues with increased hydrophobicity and/or size at these positions resulted in a decrease in sensitivity; conversely, reduced hydrophobicity or tendency for α-helix formation resulted in enhanced sensitivity to the compound. This indicates that these residues are located in or near a part of the h-region essential for translocon interaction. The h-region has been reported to be required for SP interaction with the translocon (Mothes et al., 1998), and CAM741 could interfere at this level. Amino acid changes within the h-region of the prolactin SP have been reported to alter its association with the translocon relative to Sec61α and Sec61β, consequently affecting further processing such as SP cleavage and *N*-glycosylation of the mature domain (Rutkowski et al., 2003). We have observed that mutations in the VCAM1 SP, where hydrophobicity was increased in the CAM741-sensitive region, caused a different association with the translocon, witnessed by enhanced cross-links to Sec61α and Sec61β compared with the wt VCAM1 SP (Harant et al., 2006). Also with VEGF SP variants, a different association with the translocon was seen in chemical cross-linking experiments. Although wt VEGF showed some basal cross-links to Sec61β, the less sensitive mutant VEGF (L12I, A13I, V30C) SP-SEAP already formed clearly visible basal cross-links with Sec61β in the absence of compound, possibly as a result of more efficient translocon interaction. This demonstrates individual association of the VEGF SP variants with the Sec61 translocon and could even involve different interaction sites.

The strength or site of translocon binding may be one explanation for the individual compound sensitivities of the VEGF SP mutants. However, the less sensitive mutant VEGF (Δ2–5, V30C) SP-SEAP, which lacks the N-terminal residues 2–5 but has no mutations in the h-region, gave only low basal cross-links to Sec61β, and these only increased at the highest concentration of compound tested. This demonstrates that compound sensitivity can be reduced despite the presence of an intact h-region. Thus, this segment seems to mediate translocon interaction and determines proximity to Sec61β, but compound sensitivity of the VEGF SP requires an interplay between the n- and h-regions, which could argue for a specific conformational requirement for optimal inhibition.

During the translocation process, transmembrane domains can acquire a limited degree of protein folding (such as formation of an α-helix) and, depending on the features of the transmembrane segment, folding can occur already inside the ribosome (Mingarro et al., 2000; Woolhead et al., 2004). Certain mutations in the h-region of the VEGF SP could support formation of a stabilized helix, which may enhance efficiency in translocon binding. In contrast, introduction of residues with helix-breaking potential, such as glycine or proline, could decrease helix formation propensities. Model SPs have been shown to initially insert with the N terminus facing toward the luminal side, followed by a reorientation with growing chain lengths. Such a dynamic reorientation has been suggested to occur more easily with unstable or kinked helices rather than stabilized helices (Rösch et al., 2000; Goder and Spiess, 2003). Alterations in helix formation propensity and thus enhanced flexibility of the VEGF SP within the translocon could be another reason for increased sensitivity to translocation inhibition by CAM741.

Although some of the VEGF SP mutants showed higher sensitivity to inhibition by CAM741, they showed very little response to the derivative NFI028, which, however, is fully active against VCAM1. Only the mutant VEGF (L12G, A13G) SP-SEAP showed some sensitivity to NFI028, although a much higher concentration of the compound was required for inhibition compared with VCAM1, indicating that specific features of the VEGF SP account for the poor response to NFI028. Although currently a direct binding of compound to the SP cannot be fully excluded, from our data it seems more likely that there exists a competition between the compound and SP for binding to a specific site in the translocon required to initiate the translocation process. However, as shown previously, the compound does not prevent SP binding to the translocon but may rather force the SP into a position where luminal translocation cannot occur, resulting in synthesis of the growing polypeptide chains toward the cytosolic side (Besemer et al., 2005; Harant et al., 2006). According to this hypothesis, both CAM741 and NFI028 could interact with the translocon, although binding of NFI028 would be weaker. The compounds could compete with the VCAM1 SP, which interacts with the translocon inefficiently, whereas the VEGF SP binds more efficiently and cannot be competed by NFI028. This assumption is supported by the observation that mutants of the VCAM1 SP with only slightly reduced sensitivities to CAM741 had much lower sensitivities to NFI028 (H. Harant, unpublished observations). However, the similarity in CAM741 sensitivity coupled with the large difference in NFI028 sensitivity of the VCAM1 and VEGF (L12G, A13G) SP argues against this model and suggests that not only competition but also additional SP-dependent features contribute to this selectivity.

The logical next step therefore will be the analysis of several SPs that show different degrees of sensitivity to CAM741 and the study of their association with the translocon. In addition, it would be interesting to evaluate whether insensitive SPs can be converted into sensitive ones by introduction of mutations based on our findings.

TABLE 3

Sensitivity of the VCAM1 (Δ2–10) SP and VEGF SP mutants to inhibition by NFI028

HEK293 cells were transfected with different SP-SEAP fusion constructs and incubated with increasing concentrations of NFI028. Twenty-four hours after transfection, supernatants were harvested and analyzed for alkaline phosphatase activity. Results shown are IC_{50} values from at least three independent experiments performed in triplicate. Mutations are indicated by double underlining, the cleavage site is single-underlined, and amino acid residues of the VCAM1 mature region are italic.

Signal Peptide	Sequence	NFI028 IC_{50}
		nM
VCAM (Δ 2–10)	MASNILWIMFAASQA-*FKIE*	14.3 ± 2.3
VEGF	MNFLLSWVHWSLALLLYLHHAKWSQA	>10,000
VEGF (Y17P)	MNFLLSWVHWSLALLL<u>P</u>LHHAKWSQA	3234 ± 1446
VEGF (A13P)	MNFLLSWVHWSL<u>P</u>LLLYLHHAKWSQA	4984 ± 2343
VEGF (L12G, A13G)	MNFLLSWVHWS<u>GG</u>LLLYLHHAKWSQA	464 ± 53

Acknowledgments

We thank Roland Reuschel, Waltraud Mayer-Granitzer, and Eva-Marie Haupt for sequencing and Christiane Dascher-Nadel for generation of the VEGF constructs and SP-SEAP fusion constructs. We also thank Siegfried Höfinger, Piroska Devay, and Markus Jaritz for helpful discussions.

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