THE TRANSLOCATION INHIBITOR CAM741 INTERFERES WITH VASCULAR CELL ADHESION MOLECULE 1 SIGNAL PEPTIDE INSERTION AT THE TRANSLOCON

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The cyclopeptolide CAM741 selectively inhibits cotranslational translocation of vascular cell adhesion molecule 1 (VCAM1), a process which is dependent on its signal peptide. In this study we identified the C-terminal (c-) region upstream of the cleavage site of the VCAM1 signal peptide as most critical for inhibition of translocation by CAM741, but full sensitivity to the compound also requires residues of the hydrophobic (h-) region and the first amino acid of the VCAM1 mature domain. The murine VCAM1 signal peptide, which is less susceptible to translocation inhibition by CAM741, can be converted into a fully sensitive signal peptide by two amino acid substitutions identified as critical for compound sensitivity of the human VCAM1 signal peptide. Using cysteine substitutions of non-critical residues in the human VCAM1 signal peptide and chemical crosslinking of targeted short nascent chains we show that, in the presence of CAM741, the N- and C-terminal segments of the VCAM1 signal peptide could be crosslinked to the cytoplasmic tail of Sec61β, indicating altered positioning of the VCAM1 signal peptide relative to this translocon component. Moreover, translocation of a tag fused N-terminal to the VCAM1 signal peptide is selectively inhibited by CAM741. Our data indicate that the compound inhibits translocation of VCAM1 by interfering with correct insertion of its SP into the translocon.

Amino-terminal, cleavable signal peptides (SP) mediate protein translocation to the mammalian endoplasmic reticulum (ER) by directing nascent polypeptide chains, emerging from the ribosome, to the membrane of the ER (1-6). SPs consist of three segments, a frequently positively charged N-terminus (n-region), a central hydrophobic segment (h-region) and a more polar C-terminal part (c-region) which contains the site for SP cleavage by the signal peptidase complex (SPC). Apart from these general features, SPs display little conservation in their primary sequences (7;8). Once the SP emerges from the translating ribosome, it is recognized by the signal recognition particle (SRP), which then guides the ribosome-nascent chain (NC) complex to the ER membrane via binding to the SRP receptor (SR). The ribosome-NC complex is then transferred to the Sec61 translocon, a protein conducting channel composed of the α, β and γ subunits, embedded in the ER membrane (1-6;9-22). The initial binding between the ribosome-NC complex and Sec61 is weak, but after a critical length of the translated NC, a tight, salt-resistant complex between ribosome-NC and Sec61 is formed (23;24). Accessory components, such as the translocating chain-associated membrane protein (TRAM) and the translocon-associated protein (TRAP) complex, are required for translocation of some substrates (25-30). After formation of the tight seal between ribosome-NC and translocon, the channel opens towards the lumen of the ER, allowing the NC to translocate to the luminal side of the ER, where SP cleavage and subsequent N-glycosylation takes place. These steps are controlled by the SP, and it has been suggested that different features are responsible for interaction with the SRP, insertion into the translocon and opening of the channel towards the luminal side (14;17;19;23;31-33). Moreover, SPs also contain information for further processing of the mature domain, such as the timing and efficiency of N-glycosylation and SP cleavage (34). We have recently reported that the cyclopeptolide CAM741, a derivative of the natural compound Hun-7293, can selectively inhibit cotranslational translocation of vascular cell adhesion molecule 1 (VCAM1), an action...
which is dependent on parts of the SP of VCAM1. The compound does not inhibit targeting of the VCAM1 nascent chains to the translocon, but although a salt-resistant tight complex is formed, translocation to the luminal side of the ER is prevented (35). Concurrently, Garrison et al. reported that the synthetic compound "cotransin", which has a structure very similar to CAM741, inhibits VCAM1 translocation and demonstrated that the inhibition involves the Sec61 channel but not accessory factors, such as TRAM, TRAP, and the Sec62/63 complex (36). CAM741 thus represents a highly interesting novel tool to investigate molecular events during cotranslational translocation.

To gain more insight into the action of the compound, we analysed by systematic mutagenesis the specific features of the VCAM1 SP contributing to the sensitivity to CAM741. We demonstrate here that the amino-acid residues within the c-region are critical for sensitivity to CAM741. In particular, the residues Ala20 and Ala21 upstream of the cleavage site have been identified as key residues. Together with residues of the h-region, such as Met18, and the first amino acid residue of the mature domain Phe25, they mediate full sensitivity to CAM741. As the compound does not prevent targeting of the VCAM1 NCs to the translocon, we studied the fate of the truncated VCAM1 SP in the presence of CAM741 at early post-targeting stages of the translocation process. We show that CAM741 alters the positioning of the VCAM1 SP relative to the translocon component Sec61β, indicating incorrect insertion of the SP into the translocon. We further demonstrate that translocation of a tag fused N-terminal to the VCAM1 SP, is inhibited by the compound, supporting the notion that CAM741, in a selective fashion, alters the positioning of the VCAM1 SP at the translocon.

Experimental Procedures

**Compound** - CAM741 was dissolved in DMSO and stored at -20°C.

**Plasmid constructions** - The wild-type (wt) VCAM1 construct has been described (35). The SP-secreted alkaline phosphatase (SEAP) fusion constructs were generated by PCR and subcloned into the pcDNA3.1 vector (Invitrogen). These encode the VCAM1 SP plus additional four amino acids of the VCAM1 mature region fused to the SEAP mature domain. The minimized version VCAM1 (Δ2-10) SP lacks the amino acids 2-10 of the VCAM1 SP and also contains the four amino acids of the VCAM1 mature region, fused to the SEAP mature domain. The numbering of the amino-acid residues in the truncated VCAM1 (Δ2-10) SP-SEAP construct refers to the full-length VCAM1 SP. The SEAP fusions of the mouse VCAM1 SP plus additional four amino-acid residues of the mature region, all VCAM1 SP mutants and constructs encoding fusions of the N-terminal tag to the SP were generated by PCR and subcloned into pcDNA3.1. The truncated VCAM1 cDNA, encoding the 112 amino-acid residues subcloned into pcDNA3.1, has been described (35). The truncated VCAM1 cDNA encoding 182 amino-acid residues was generated by PCR and subcloned into pcDNA3.1. The IL-6 cDNA and the construct encoding the N-terminal tag fused to the IL-6 coding region were generated by PCR and subcloned into pcDNA3.1. All constructs were confirmed by sequencing.

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 either linearized by digestion with Bst EII (encoding 146 amino-acid residues SEAP mature domain) or Bam HI (encoding 54 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 (Promega).

**Transient transfection of HEK293 cells** - HEK293 cells were cultivated in DMEM supplemented with 10% FCS and passaged twice a week. For transfection, 1.5x10^4 cells were seeded per well of a 96-well plate and transfected with 0.2 µg/well plasmid DNA and 0.5 µl/well Superfect (Qiagen) and treated with increasing concentrations of CAM741. Supernatants were harvested after 24 hrs and analysed for SEAP secretion using the AttoPhos reagent (Promega) and fluorescence was recorded using the SPEKTRAmos GEMINI XS (Molecular Devices).

**In vitro translocation experiments** - In vitro translation, targeting and translocation assays were performed with truncated RNAs using rabbit reticulocyte lysate, canine pancreatic microsomal...
membranes (Promega) and [\(^{35}\)S]-methionine (Amersham), in the presence of CAM741 or DMSO (vehicle control) in a volume of 25 µl. Targeting was performed for 30 min at 26°C. Release of the NCs from the ribosome was induced after 10 min targeting at 26°C followed by treatment with 3.4 µl 4 M potassium acetate, 2.6 µl 20 mM magnesium acetate and 3 µl puromycin (36 mM) for 20 min at 26°C. The reaction mix was then diluted with 75 µl of 250 mM sucrose, 150 mM potassium acetate, 40 mM HEPES (pH 7.3), 5 mM magnesium acetate, 2 mM dithiothreitol and layered on a cushion of 100 µl 500 mM sucrose, 500 mM potassium acetate, 40 mM HEPES (pH 7.3), 2 mM magnesium acetate and 2 mM dithiothreitol. In some cases, volume was doubled. Centrifugation was performed in a Beckman TLA120 rotor or a Sorvall S100AT3 rotor at 50,000 rpm for 4 min, or for larger volumes in a Sorvall S140AT rotor at 54,000 rpm for 5 min. For proteinase K digestion, pelleted membranes were resuspended in 40 mM HEPES, pH 7.3, 200 mM sucrose, 50 mM potassium acetate, 5 mM magnesium acetate, 2 mM calcium chloride and treated with proteinase K (12.5 µg/ml; Roche) for 30 min on ice. For deglycosylation, sedimented membranes were lysed with 10 µl lysis buffer (0.05% deoxycholic acid in PBS, 250 mM sodium chloride, 10 µl/ml IGEPAL (Sigma-Aldrich)). Then 8 µl PBS, 1 µl 500 mM EDTA and 1 µl 10%SDS were added, and samples heated at 95°C for 1 min. Then, 2 µl Triton X-100 and 3 µl N-Glycosidase F (Roche) were added and samples incubated at 37°C for 60 min. Chemical crosslinking was carried out as described (35). Immunoprecipitations were then performed with a polyclonal Sec61β or Sec61α antiserum (Upstate). Proteins were separated on Excel Gel™ SDS 8-18% or, where indicated, high resolution Excel Gel™ SDS 12-14% gels (Amersham) and fixed and dried gels were exposed to X-ray films.

RESULTS

Identification of the key amino-acid residues of the human VCAM1 SP contributing to CAM741 sensitivity

Fusion constructs of the VCAM1 SP and the mature region of secreted alkaline phosphatase (SEAP) were used to investigate the residues of the VCAM1 SP critical for sensitivity to CAM741. Previous analysis of the VCAM1 SP showed that the amino acids 2-10 are not required for translocation and are also dispensable for the compound effect, but that the first amino acid of the VCAM1 mature domain is required for full sensitivity (35). To narrow down the residues involved in the action of CAM741, we utilized the minimized VCAM1 SP, which lacks the amino acids 2-10, but contains four additional amino acids (Phe-Lys-Ile-Glu) of the VCAM1 mature domain fused to the SEAP mature domain (VCAM1 (Δ2-10) SP-SEAP). Transient transfections of HEK293 cells with this construct showed, that CAM741 inhibited SEAP release with an IC\(_{50}\) of 9 nM (Table 1). Mutation of amino acids within the N-terminal region or in the central hydrophobic region of VCAM1 (Δ2-10) SP-SEAP in most instances had no effect on compound sensitivity. However, there was some decrease in sensitivity observed for the mutants VCAM1 (Δ2-10; S12G) SP-SEAP and VCAM1 (Δ2-10; M18I) SP-SEAP, while the double mutant VCAM1 (Δ2-10; W16L, M18I) SP-SEAP showed greatly reduced sensitivity to CAM741 (Table 1).

We reported previously that the first amino acid (Phe25) of the VCAM1 mature region is required for full sensitivity (35). As expected from this earlier observation, changing the second or third amino acid of the VCAM1 mature domain did not further affect sensitivity to CAM741. On the other hand, replacing Phe25 by glycine (F25G) decreased sensitivity to the compound (Table 1). Although Phe25 contributes to CAM741 sensitivity, the cleavage site itself could be altered without loss in sensitivity. Replacement of Ser22 by Val (S22V), or the complete cleavage site by Val-His-Gly (S22V, Q23H, A24G), even increased sensitivity to CAM741. However, certain mutations in the c-region upstream of the cleavage site clearly reduced sensitivity to CAM741. When Ala20 was changed to valine (A20V), sensitivity was slightly decreased, while an isoleucine in this position (A20I) further decreased sensitivity to the compound (Table 1). A clear reduction in sensitivity resulted from changes of Ala21 into valine (A21V), which further decreased by the additional amino acid change at Met18 (M18I, A21V). The greatest loss in sensitivity was observed when both Ala20 and
Ala21 were changed into Val (A20V, A21V); secretion of SEAP was inhibited by CAM741 with an IC₅₀ of 600 nM (Table 1).

Transient transfections were also performed with fusion constructs of the full-length (FL) VCAM1 SP plus the four additional amino acids of the VCAM1 mature region fused to the SEAP mature domain. The sensitivity of the full-length VCAM1 SP to CAM741 was slightly lower compared to the truncated VCAM1 (∆2-10) SP-SEAP construct. In addition, the mutant VCAM1 (FL; M18I, A21V) SP-SEAP, also displayed reduced sensitivity to CAM741 comparable to the corresponding truncated construct VCAM1 (∆2-10; M18I, A21V) SP-SEAP, confirming the importance of these residues in drug sensitivity (Table 1).

These data indicate that the residues Ala20 and Ala21 upstream of the cleavage site together with Met18 of the h-region, and Phe25, the first amino-acid residue of the mature domain, are required for full sensitivity to CAM741.

Conversion of the mouse VCAM1 SP into a fully CAM741-sensitive SP by amino acid substitutions present in the human VCAM1 SP

Inhibition of murine VCAM1 expression requires higher concentrations of CAM741 compared to human VCAM1 (unpublished observations). Here we show that transient transfections of HEK293 cells with a construct containing the full-length mouse VCAM1 SP plus four additional residues of the mature domain fused to SEAP (mouse VCAM1 (FL) SP-SEAP) resulted in clearly reduced sensitivity to CAM741 (Table 1).

The mouse VCAM1 SP has several amino-acid residues different from the human VCAM1 SP, and contains a valine at the critical position 21 and a leucine at position 18. We therefore replaced these amino-acid residues by the ones present in the human counterpart and tested these constructs for sensitivity to CAM741 by transient transfection experiments. While conversion of Leu18 to Met (L18M) in the mouse VCAM1 SP resulted in some increase in sensitivity to CAM741, conversion of Val21 to Ala (V21A) clearly enhanced sensitivity to the compound. However, conversion of both residues, in mouse VCAM1 (L18M, V21A) SP, yielded a construct with almost the same sensitivity to CAM741 as the full-length human VCAM1 SP. The results from this "humanized" mouse VCAM1 SP confirm the importance of these residues in determining sensitivity to CAM741 (Table 1).

Differential sensitivity of VCAM1 SP mutants to inhibition by CAM741 occurs at the level of cotranslational translocation

We determined whether the differential response of the VCAM1 SP mutants to CAM741 observed in the cellular assay is reflected at the level of cotranslational translocation. VCAM1 SP-SEAP fusion constructs were linearized with Bst EII to create truncated RNAs lacking a stop codon. These were then used for in vitro translation in the presence of canine pancreatic microsomal membranes to generate translocation intermediates of SP + 146 amino-acid residues SEAP mature domain (Fig. 1A). The SEAP mature domain contains a glycosylation site at position 122 which allows detection of translocated NCs after release from the ribosome by high salt/puromycin treatment (Fig. 1A, B). In vitro translocation of the truncated VCAM1 (∆2-10) SP-SEAP fusion construct showed that the translocated, glycosylated fragment was protected from digestion by added protease, and degraded only after lysis of the microsomal membranes with Triton X-100. Another faster migrating fragment, which was also protected from added protease, resembles the non-glycosylated form with the SP cleaved off (Fig. 1B, left upper panel). Glycosylation was confirmed by treatment of translocated NCs with endoglycosidase F (Fig. 1B, right panel). However, in the presence of CAM741, only a single, protease-sensitive fragment was observed, demonstrating inhibition of translocation of the VCAM1 (∆2-10) SP-SEAP fusion construct by CAM 741 (Fig. 1B, left upper panel).

The dose-dependent effect of CAM741 on translocation of several constructs was then further analysed. Translocation of the fusion partner SEAP, under control of its own SP, was not inhibited by CAM741 up to a concentration of 1 µM (Fig. 1C) (35). However, translocation of the VCAM1 (∆2-10) SP-SEAP fusion construct was dose-dependently inhibited by CAM741. The constructs VCAM1 (∆2-10; A21V) SP-SEAP, VCAM1 (∆2-10; M18I, A21V) SP-SEAP and
VCAM1 (∆2-10; A20V, A21V) SP-SEAP required higher concentrations of CAM741 for inhibition, which is in agreement with the data obtained from the transient transfection experiments (Table 1, Fig. 1C). Moreover, the differential compound sensitivity of the full-length VCAM1 SP, the mouse VCAM1 SP and its mutations determined by transient transfection assays, could be confirmed by the in vitro translocation experiments (Table 1, Fig. 1C).

To exclude any effect of the SEAP fusion partner in the in vitro translocation experiments, we also analysed 182 amino-acid residues translocation intermediates of wt VCAM1 (Fig. 1A). As there is no glycosylation site present within this chain length, SP cleavage and protection from exogenous protease indicates correct translocation (Fig. 1B, lower panel). As shown in Fig. 1C, SP cleavage of the wt VCAM1 was inhibited by CAM741 at low concentrations, while inhibition of SP cleavage of the less sensitive mutant VCAM1 (M18I, A21V) required higher concentrations of the compound (Fig. 1C). Taken together, the differential sensitivity of the VCAM1 SP mutants to inhibition by CAM741 occurs at the level of translocation.

CAM741 alters positioning of the VCAM1 SP relative to the translocon component Sec61β

The identification of critical amino-acid residues which contribute to the sensitivity to CAM741 and those which do not affect sensitivity, allowed us to replace single non-critical amino acids with cysteine residues to analyse the positioning of the VCAM1 SP within the translocon by chemical crosslinking experiments.

First, we replaced amino-acid residues by cysteines in the truncated VCAM1 (∆2-10) SP fused to the SEAP mature region and analysed these SP mutants by transient transfections in HEK293 cells, to determine whether these substitutions affect translocation or sensitivity to CAM741. As outlined in Table 2, none of the single cysteine residues greatly affected sensitivity to CAM741. Second, short NCs (VCAM1 (∆2-10) SP + 54 amino-acid residues SEAP mature domain) of sufficient length for targeting were created. At this chain length, the SEAP mature domain does not contain any cysteine residues, and therefore the only cysteine residue is provided by the mutated VCAM1 SP. These short NCs were used for targeting to microsomal membranes in the absence or presence of CAM741 followed by chemical crosslinking with the cysteine-reactive crosslinker bis-maleimidohexane (BMH). With several, but not all cysteine mutants formation of an enhanced crosslinked product was observed in the presence of CAM741, identified by immunoprecipitation to contain Sec61β. However, in the absence of CAM741, only weak crosslinks to Sec61β were observed (Fig. 2). In particular, CAM741-induced enhanced crosslinks to Sec61β were observed when amino-acid residues within the N-terminal part of the minimized VCAM1 (∆2-10) SP, such as Ala11 or Asn13, were replaced by cysteines. In addition, upon introduction of cysteine residues instead of Ala20 within the c-region, Ala24 within the cleavage site, or Lys26, the second residue of the VCAM1 mature region, enhanced crosslinking to Sec61β was observed. One exception was the cysteine substitution of Ser22, the first residue of the cleavage site, which did not form an enhanced crosslink to Sec61β, but was sensitive to CAM741. Moreover, replacement of amino-acid residues within the hydrophobic region, such as Leu15 or Met18, by cysteine residues, did not result in enhanced crosslinks to Sec61β (Fig. 2). This suggests that these amino acids within the VCAM1 SP may be inaccessible to crosslinker or facing away from Sec61β even in the presence of CAM741 and therefore could not crosslink. As the single cysteine residue of Sec61β is located at the cytosolic side, these results suggest that, in the presence of compound, the VCAM1 SP is improperly attached to the Sec61 translocon, with both the N-terminal and C-terminal segments facing towards the cytosolic side as depicted in the scheme (Fig. 2).

VCAM1 SP mutants with decreased sensitivity to inhibition by CAM741 are differently associated with the translocon

To determine whether the appearance of the Sec61β crosslinks also occurs with less sensitive VCAM1 (∆2-10) SP mutants, lysine at position 26 was substituted by cysteine in the mutants VCAM1 (∆2-10; M18I, A21V) SP-SEAP and VCAM1 (∆2-10; A20V, A21V) SP-SEAP.
Although SP cleavage was not observed at the chain length used, the cysteine substitution at position 26 was chosen to exclude that the lack of Sec61β crosslinks in the absence of CAM741 could derive from SP cleavage. These VCAM1 SP mutants were then tested by transient transfections of HEK293 cells for their sensitivity to CAM741 (Table 2), and short targeted NCs (SP + 54 amino-acid residues SEAP mature domain) were subjected to chemical crosslinking with BMH. With the fully sensitive VCAM1 (Δ2-10; K26C) SP-SEAP, the Sec61β crosslink was clearly visible at a concentration of 10 nM CAM741, which further increased with higher concentrations of the compound, while showing only marginal crosslinks with Sec61β in the absence of compound (Fig. 3). However, although the less sensitive VCAM1 SP mutants started to form enhanced Sec61β crosslinks only at higher concentrations of compound, there were already basal Sec61β crosslinks formed also in the absence of compound, in particular with the mutant VCAM1 (Δ2-10; A20V, A21V) SP-SEAP (Fig. 3). One possible explanation would be that the less sensitive mutants have been differentially inserted into the translocon or, in contrast to the wt VCAM1 SP, show enhanced binding to the translocon channel and therefore also to Sec61β (Fig. 3). This is further supported by the observation that chemical crosslinking of the targeted NCs showed formation of a higher molecular weight crosslink with the less sensitive mutants VCAM1 (Δ2-10; M18I, A21V, K26C) SP-SEAP and VCAM1 (Δ2-10; A20V, A21V, K26C) SP-SEAP. However, this crosslink was only marginally formed with the highly sensitive VCAM1 (Δ2-10; K26C) SP-SEAP. By immunoprecipitation, the presence of Sec61α in this crosslinked product was identified. These data indicate that the less sensitive VCAM1 SP mutants are associated differently and even more efficiently with the translocon compared to the fully sensitive wt VCAM1 SP (Fig. 3).

Enhanced formation of the VCAM1 SP-Sec61β crosslink by CAM741 requires the presence of compound during targeting

The effect of CAM741 on formation of the Sec61β crosslink with targeted VCAM1 (Δ2-10; K26C) SP-SEAP NCs was studied when applied at different time points. The maximal level of VCAM1 SP-Sec61β crosslink formed only when the reaction was preincubated with CAM741, and was reduced when the compound was added 15 min after initiation of targeting. However, when the compound was applied 30 min after initiation of targeting, no enhanced VCAM1 SP-Sec61β crosslink was formed (Fig. 4A). This indicates that, once the VCAM1 SP has inserted correctly into the Sec61 translocon, CAM741 is unable to alter its positioning when applied after this step.

Based on this observation, we further analysed the effect of the compound on VCAM1 translocation when applied after targeting but before release of the NCs from the ribosome by high salt/puromycin treatment. Wild-type VCAM1 NCs (representing the full length VCAM1 SP with its mature domain) of 112 amino-acid residues were used, as in our earlier study it was shown that at this chain length, the targeted VCAM1 NCs were protease-protected, but no SP cleavage was observed during targeting. However, after release of the NCs from the ribosome, SP cleavage was detected (35). When CAM741 was added to the reaction before targeting, SP cleavage was inhibited after release of the NCs with high salt/puromycin (Fig. 4B). However, when CAM741 was added 30 min after initiation of targeting followed by high salt/puromycin-release, the compound could not inhibit translocation, as evident from SP cleavage (Fig. 4B). Taken together, these observations suggest that CAM741 alters the positioning of the VCAM1 SP at the translocon site and that the compound has to be present during the targeting process.

CAM741 prevents translocation of an N-terminal tag fused to the VCAM1 SP

It has been shown that SPs can enter the translocon in a head-on insertion with the N-terminal end first facing the lumenal side. In addition, inserted SPs are capable of reorientation within the translocon channel (34;37). To determine whether the VCAM1 SP could also insert in such a way, a 17 amino-acid residues tag was fused N-terminally to the full-length VCAM1 SP (N-tag) and its mature domain. This tag was designed according to the construct used by Heinrich et al. for studies on transmembrane domain insertions (38). It contains a diagnostic...
glycosylation site at the N-terminal end as shown in Fig. 5A, and glycosylation therefore allows detection of N-terminal translocation. Targeting with the N-terminally tagged VCAM1 NC of 112 amino-acid residues in the presence of microsomal membranes resulted in the formation of a slower migrating glycosylated and a non-glycosylated form, showing translocation of the N-terminal tag to the luminal side of the ER (Fig. 5B, upper middle panel). However, glycosylation was observed only in untreated reactions but not when CAM741 was present, demonstrating that the compound also prevents N-terminal translocation of the tag (Fig. 5B, upper middle panel). This compound effect was specific for the VCAM1 SP, as it had no effect on translocation of the N-terminal tag fused to a 67 amino-acid residues interleukin-6 (IL-6) translocation intermediate (Fig. 5B, lower middle panel). Resistance of the IL-6 SP to translocation inhibition by CAM741 was further confirmed by transient transfections of HEK293 cells (data not shown). As additional controls, the constructs without the N-terminal tag were also subjected to targeting, demonstrating that no other N-glycosylation sites are present. Moreover, glycosylation of the N-terminally tagged fusion constructs was confirmed by treatment with endoglycosidase F (Fig. 5B). The effect of CAM741 on translocation of the N-terminal tag fused to the VCAM1 fragment was dose-dependent (Fig. 5C).

To determine whether the tag affects translocation in living cells, the N-terminally tagged full-length (FL) VCAM1 SP plus four additional amino acids of the VCAM1 mature domain was fused to the SEAP mature domain. This construct was driving release of SEAP by transfected HEK293 cells, and was fully sensitive to inhibition by CAM741 (Fig. 5D). Additionally, targeted short nascent chains (N-tag VCAM1 (FL) SP plus 54 amino-acid residues SEAP mature domain) showed a comparable picture as N-tagged VCAM1 NCs (compare Fig. 5B and D).

Goder and Spiess have reported that SP reorientation occurs with growing chain lengths (37). We therefore analysed at which length of the VCAM1 NCs, glycosylation of the N-terminal tag can occur. With the shortest construct tested (57 amino-acid residues VCAM-1), formation of small amounts of glycosylated product was observed. Interestingly, at this chain length, although only weakly glycosylated, exposure of the N-terminal tag to the lumen was not inhibited by CAM741. However, at a chain length of 67 amino-acid residues, glycosylation largely disappeared when CAM741 was present (Fig. 5E).

These results show that the VCAM1 SP at the shortest chain length tested, has initially inserted in a similar way both, in the vehicle- and CAM741-treated reactions. However, only in the absence of compound, further chain elongation results in N-terminal translocation of the tag.

**DISCUSSION**

We have recently described that CAM741, a derivative of the natural fungus-derived cyclopeptolide Hun-7293, can selectively inhibit the process of cotranslational translocation of VCAM1 and that this inhibition is dependent on the SP of VCAM1 (35). Very similar conclusions were made by Garrison et al., who reported inhibition of VCAM1 translocation by cotransin, a compound of similar structure (36). However, the features of the VCAM1 SP conferring this selectivity remained to be identified.

In this paper we describe a mutagenesis study of the VCAM1 SP to identify the key amino-acid residues involved in the inhibitory action of CAM741. We show that the residues within the c-region upstream of the SP cleavage site contribute to sensitivity to CAM741. In particular, Ala21 is critical, as mutation of this residue into valine along with other amino-acid substitutions caused most severe reduction in sensitivity to the compound. The importance of this residue was further confirmed by "humanizing" the less sensitive mouse VCAM1 SP into a sensitive SP through changing Val21 of the murine SP into alanine. Although the region most critical for translocation inhibition by CAM741 is located in the c-region upstream of the cleavage site of the VCAM1 SP, optimal sensitivity also requires certain residues of the h-region, such as Met18, and the first residue of the VCAM1 mature domain. The results from this mutation analysis indicate that these residues and probably a specific conformation of the VCAM1 SP mediate interaction with the translocon and thus the selectivity of VCAM1 translocation inhibition by CAM741 could occur via interference with this interaction.
It is evident from several studies that SPs control three steps during the process of translocation; first, the recognition by the SRP, which guides the ribosome-NC complex to the Sec61 translocon, second, the transfer of the NC to the translocon and achievement of the salt-resistant status, and third, the opening of the translocon towards the ER lumen (3;4;6). Although SPs require general features for this process, such as a certain number of hydrophobic residues, specific features of the SP appear to be required for the successful performance of individual steps in translocation. Recognition by the Sec61 complex may therefore be more stringent compared to binding to the SRP as reported for preprolactin SP deletion mutants (23). The third function of the SP, the opening of the channel towards the luminal side, has been proposed to be controlled by specific features of the SP. Kim et al. demonstrated that different amino-acid residues of the signal sequence of a prion protein were involved in targeting and in generation of the salt-resistant complex, as some mutants which crosslinked to SRP and Sec61 were defective in translocating towards the lumen of the ER (33). Similar to the behaviour described for such SP mutants, the first two steps in VCAM1 translocation, the targeting and salt-resistant binding were not impaired. However, luminal translocation was inhibited by CAM741, demonstrating that it acts on a post-targeting step (35), further suggesting that the compound exerts its action at the level of SP-translocon assembly. We now provide experimental data indicating that insertion of the VCAM1 SP at the translocon is altered by CAM741. This is evident from enhanced direct crosslinking of the VCAM1 SP to Sec61β in the presence of compound and demonstrates that also under these conditions, the VCAM1 SP is still in close proximity to the translocon although positioned differently. In particular, amino-acid residues before or after the cleavage site, which would be expected to face towards the luminal side of the ER, could be crosslinked to the single cysteine residue present in the cytoplasmic tail of Sec61β. In addition, the c-region upstream of the cleavage site is also most critical for compound sensitivity, and its close proximity to Sec61β could argue for a CAM741-induced rejection of these residues from correct translocon interaction. However, although the VCAM1 SP appears to be incorrectly inserted in the presence of CAM741, this interaction is sufficient for targeting and tight complex formation (35). Binding of SPs to the Sec61 translocon has been shown to require the h-region (39), and also in the presence of CAM741, the h-region of the VCAM1 SP may have sufficiently attached to the translocon, allowing tight complex formation. This may explain the lack of Sec61β crosslinks of cysteine substitutions of positions Leu15 or Met18 even in the presence of CAM741 and could indicate that this region is inserted in the translocon and therefore not accessible to crosslinking.

With two less sensitive VCAM1 SP mutants, higher concentrations of CAM741 were required for translocation inhibition and enhanced crosslinking to Sec61β. However, in contrast to the wt VCAM1 SP, where only marginal Sec61β crosslinks were observed in the absence of compound, basal crosslinks to Sec61β and also Sec61α were visible. This indicates that their insertion is different from wt VCAM1 SP and that they may interact with the translocon more efficiently which could make them less susceptible to inhibition by CAM741.

It has been shown by chemical crosslinking that a close proximity to Sec61β is one of the early events during TM domain integration (40;41). The crosslinks with Sec61β disappear once the TM domain moves laterally into the membrane, as evident from enhanced lipid crosslinks and glycosylation of a tag N-terminal to the TM domain (38;42). Based on the observation that in the presence of CAM741, the VCAM1 SP is located at the translocon but is incorrectly positioned, the compound could prevent correct partitioning of the SP at the translocon/membrane interface or affect the SP orientation within the translocon. Therefore we analysed the effect of CAM741 on glycosylation of the N-terminally tagged VCAM1 SP. Our results indicate, that initially the N-tagged VCAM1 SP has attached to the translocon in a similar way both, in the absence or presence of compound. However, with growing chain length, translocation of the N-tag is only operative in the absence of CAM741, supporting the concept that correct positioning of the VCAM1 SP within the translocon is prevented by the compound.
Our results from the crosslinking experiments and N-terminal translocation therefore allow two interpretations: the wt VCAM1 SP is inefficiently bound to the translocon, and the compound displaces or removes the VCAM1 SP from a specific binding site, forcing it into a different position. This concept is supported by the results described by Garrison et al. who showed that cotransin can prevent the productive interaction of VCAM1 NCs with the Sec61 complex (36). Alternatively, CAM741 induces even an enhanced binding of the VCAM1 SP to an incorrect site at the translocon. The latter idea is supported by the observation that, in the presence of inhibitory concentrations of CAM741, higher amounts of labeled nascent chains can be sedimented.

The observation that the VCAM1 SP is highly sensitive to inhibition by CAM741 further raises the question, whether different interaction sites in the translocon are used by individual SPs. Examples for different translocon interactions of SPs have been provided in the literature. It has been shown that, during early events in translocation, the preprolactin SP is in close contact with Sec61α, while at later stages, when tight complex formation takes place, it is in contact with Sec61α, TRAM and lipids. However, the SP of prepro-α-factor, showed some lipid crosslinks already at shorter chain lengths (39). These data indicate that at least some SPs initially require only protein-protein interactions and move to the protein-lipid interface at later stages, but that other SPs may already move into the protein-lipid interface at shorter chain lengths (26;39;43). Thus, as SPs differ in their features necessary to control essential events, such as tight complex formation and translocation towards the luminal side or the ER (32), it is possible that SPs may indeed utilize different modes of insertion into the translocon. A more extensive comparison of SPs will be required to address this question in detail.

Further challenging issues therefore include the identification of the exact binding site of CAM741, and to extend the search for other sensitive SPs. Moreover, insights into the structural representation of the VCAM1 SP should also help to identify critical positions required for the translocation process. This should then further support the identification of possibilities for pharmacological interference with this process in a SP-selective manner.

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REFERENCES

1. Rapoport, T. A., Jungnickel, B., and Kutay, U. (1996) Annu.Rev.Biochem. 65:271-303, 271-303
2. Hegde, R. S. and Lingappa, V. R. (1997) Cell 91, 575-582
3. Matlack, K. E., Mothes, W., and Rapoport, T. A. (1998) Cell 92, 381-390
4. Johnson, A. E. and van Waes, M. A. (1999) Annu.Rev.Cell Dev.Biol. 15, 799-842
5. Stroud, R. M. and Walter, P. (1999) Curr.Opin.Struct.Biol. 9, 754-759
6. Osborne, A. R., Rapoport, T. A., and van den, B. B. (2005) Annu.Rev.Cell Dev.Biol.
7. Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997) Protein Eng 10, 1-6
8. Nielsen, H., Brunak, S., and von Heijne, G. (1999) Protein Eng 12, 3-9
9. Gilmore, R., Blobel, G., and Walter, P. (1982) J.Cell Biol. 95, 463-469
10. Gilmore, R., Walter, P., and Blobel, G. (1982) J.Cell Biol. 95, 470-477
11. Meyer, D. I., Krause, E., and Dobberstein, B. (1982) Nature 297, 647-650
12. Krieg, U. C., Walter, P., and Johnson, A. E. (1986) Proc.Natl.Acad.Sci.U.S.A 83, 8604-8608
13. Kurzchalia, T. V., Wiedmann, M., Girshovich, A. S., Bochkareva, E. S., Bielka, H., and Rapoport, T. A. (1986) Nature 320, 634-636
14. Simon, S. M. and Blobel, G. (1991) Cell 65, 371-380
15. Gorlich, D., Hartmann, E., Prehn, S., and Rapoport, T. A. (1992) Nature 357, 47-52
16. Gorlich, D., Prehn, S., Hartmann, E., Kalies, K. U., and Rapoport, T. A. (1992) Cell 71, 489-503
17. Crowley, K. S., Reinhart, G. D., and Johnson, A. E. (1993) Cell 73, 1101-1115
18. Kalies, K. U., Gorlich, D., and Rapoport, T. A. (1994) J.Cell Biol. 126, 925-934
19. Crowley, K. S., Liao, S., Worrell, V. E., Reinhart, G. D., and Johnson, A. E. (1994) Cell 78, 461-471
20. Menetret, J. F., Neuhol, A., Morgan, D. G., Plath, K., Radermacher, M., Rapoport, T. A., and Akey, C. W. (2000) Mol.Cell 6, 1219-1232
21. Morgan, D. G., Menetret, J. F., Neuhol, A., Rapoport, T. A., and Akey, C. W. (2002) J.Mol.Biol. 324, 871-886
22. Menetret, J. F., Hegde, R. S., Heinrich, S. U., Chandramouli, P., Ludtke, S. J., Rapoport, T. A., and Akey, C. W. (2005) J.Mol.Biol. 348, 445-457
23. Jungnickel, B. and Rapoport, T. A. (1995) Cell 82, 261-270
24. Song, W., Raden, D., Mandon, E. C., and Gilmore, R. (2000) Cell 100, 333-343
25. High, S., Martoglio, B., Gorlich, D., Andersen, S. S., Ashford, A. J., Giner, A., Hartmann, E., Prehn, S., Rapoport, T. A., and Dobberstein, B. (1993) J.Biol.Chem. 268, 26745-26751
26. Mothes, W., Prehn, S., and Rapoport, T. A. (1994) EMBO J. 13, 3973-3982
27. Nicchitta, C. V., Murphy, E. C., Haynes, R., and Shelnness, G. S. (1995) J.Cell Biol. 129, 957-970
28. Voigt, S., Jungnickel, B., Hartmann, E., and Rapoport, T. A. (1996) J.Cell Biol. 134, 25-35
29. Hegde, R. S., Voigt, S., Rapoport, T. A., and Lingappa, V. R. (1998) Cell 92, 621-631
30. Fons, R. D., Bogert, B. A., and Hegde, R. S. (2003) J.Cell Biol. 160, 529-539
31. Hanein, D., Matlack, K. E., Jungnickel, B., Plath, K., Kalies, K. U., Miller, K. R., Rapoport, T. A., and Akey, C. W. (1996) Cell 87, 721-732
32. Rutkowski, D. T., Lingappa, V. R., and Hegde, R. S. (2001) Proc.Natl.Acad.Sci.U.S.A 98, 7823-7828
33. Kim, S. J., Mitra, D., Salerno, J. R., and Hegde, R. S. (2002) Dev.Cell 2, 207-217
34. Rutkowski, D. T., Ott, C. M., Polansky, J. R., and Lingappa, V. R. (2003) J.Biol.Chem. 278, 30365-30372
35. Besemer, J., Harant, H., Wang, S., Oberhauser, B., Marquardt, K., Foster, C. A., Schreiner, E. P., de Vries, J. E., Dascher-Nadel, C., and Lindley, I. J. (2005) Nature 436, 290-293
36. Garrison, J. L., Kunkel, E. J., Hegde, R. S., and Taunton, J. (2005) Nature 436, 285-289
37. Goder, V. and Spiess, M. (2003) EMBO J. 22, 3645-3653
38. Heinrich, S. U., Mothes, W., Brunner, J., and Rapoport, T. A. (2000) Cell 102, 233-244
39. Mothes, W., Jungnickel, B., Brunner, J., and Rapoport, T. A. (1998) J.Cell Biol. 142, 355-364
40. Laird, V. and High, S. (1997) J.Biol.Chem. 272, 1983-1989
41. Ismail, N., Crawshaw, S. G., and High, S. (2006) J.Cell Sci. 119, 2826-2836
42. Heinrich, S. U. and Rapoport, T. A. (2003) EMBO J. 22, 3654-3663
FOOTNOTES

1The abbreviations used are: BMH, bis-maleimido-hexane; IL, interleukin; NC, nascent chain; SEAP, secreted alkaline phosphatase; SP, signal peptide; SR, signal recognition particle receptor; SRP, signal recognition particle; TM, transmembrane; TRAM, translocating chain-associated membrane protein; TRAP, translocon-associated protein; VCAM1, vascular cell adhesion molecule 1; wt, wild-type;

FIGURE LEGENDS

Fig. 1 Differential sensitivity of VCAM1 SP mutants to translocation inhibition by CAM741
A, Schematic representation of the constructs used. B, in vitro translocation of a fusion construct of the VCAM1 (Δ2-10) SP and the 146 amino-acid residues SEAP mature domain (upper left panel), or 182 amino-acid residues wt VCAM1 NCs (lower panel) in the absence or presence of 1 µM CAM741. Translocation reactions were either left untreated, treated with proteinase K, or proteinase K and 1% Triton X-100. Deglycosylation of translocated VCAM1 (Δ2-10) SP-SEAP with endoglycosidase F (Endo F; right panel). C, in vitro translocation of fusion constructs of VCAM1 SP mutants and the 146 amino-acid residues SEAP mature domain, or of 182 amino-acid residues wt VCAM1 NCs or VCAM1 NCs containing the mutation M18I and A21V in the SP, in the presence of increasing concentrations of CAM741. Arrow, glycosylated NCs, star, non-processed NCs, arrowhead, NCs with the SP cleaved off.

Fig. 2 CAM741 alters positioning of the VCAM1 SP relative to Sec61β
Schematic representation of the constructs used, stars underlying the amino-acid sequence of the truncated VCAM1 SP indicate the positions of the single cysteine residues, the cleavage site is underlined. In vitro targeting and crosslinking with BMH of the indicated VCAM1 (Δ2-10) SP mutants fused to 54 amino-acid residues of the SEAP mature domain in the absence or presence of 1 µM CAM741 (upper panels) and immunoprecipitation with the Sec61β antiserum (lower panels). Closed circle, Sec61β crosslink, star, NCs. Model of the possible SP insertions in the absence or presence of CAM741.

Fig. 3 Less CAM741-sensitive VCAM1 SP mutants are differently associated with the translocon
Left panels: crosslinking with BMH of short targeted VCAM1 (Δ2-10; K26C) SP-SEAP NCs in the absence or presence of increasing concentrations of CAM741; middle panels: crosslinking with BMH of short targeted NCs in the absence or presence of 100 nM CAM741 and immunoprecipitation with Sec61β antiserum; right panels: crosslinking with BMH of short targeted NCs in the absence or presence of 100 nM CAM741 and immunoprecipitation with Sec61α antiserum. Closed circle, Sec61β crosslink, closed diamonds, Sec61α crosslink, star, NCs, open circle, residual peptidyl-tRNA-NCs.

Fig. 4 The inhibitory action of CAM741 requires the presence of compound during targeting
A, crosslinking with BMH and immunoprecipitation with the Sec61β antiserum of short targeted VCAM1 SP (Δ2-10; K26C) SP-SEAP NCs in the absence or presence of 1 µM CAM741, applied at the indicated time points. pre, preincubated for 30 min, 15’, applied 15 min after initiation of targeting, 30’, applied 30 min after initiation of targeting; Closed circle, Sec61β crosslink; star, NCs; B, Schematic representation of the construct used. In vitro targeting (T), translocation by high salt-puromycin release (P) and proteinase K treatment of 112 amino-acid residues wt VCAM-1 NCs in the absence of compound (control; left panel), after preincubation of membranes with 1 µM CAM741 for 30 min (preincubation; middle panel), or 1 µM CAM741 applied after 30 min of targeting, but before high salt/puromycin release (post-targeting; right panel). Star, unprocessed NCs, arrowhead, NCs with the SP cleaved off.
**Fig. 5** CAM741 inhibits N-terminal translocation of a tag fused to the VCAM1 SP.

*A* Sequences of the constructs used. The N-terminal tag is indicated in italic letters, the glycosylation site is underlined. 

*B* In vitro targeting of truncated 112 amino-acid residues VCAM1 NCs (112 aa; upper left and middle panels), or truncated 67 amino-acid residues IL-6 NCs (67 aa; lower left and middle panels) with or without the 17 amino-acid residues N-tag, in the absence or presence of 1 µM CAM741. Deglycosylation of targeted N-tagged VCAM1- or IL-6 NCs with endoglycosidase F (Endo F, right panels). 

*C* In vitro targeting of 112 amino-acid residues VCAM1 NCs fused to the 17 amino-acid residues N-tag in the presence of increasing concentrations of CAM741. 

*D* In vitro targeting of the N-terminally tagged full-length VCAM1 SP plus four amino acids of the mature region fused to SEAP in the absence or presence of CAM741. Below the IC$_{50}$ (nM) from transient transfections of HEK293 cells with this construct and treatment with increasing concentrations of CAM741 for 24 hrs. 

*E* In vitro targeting of 57 or 67 amino-acid residues VCAM1 NCs, both fused to the 17 amino-acid residues N-tag. Arrow, glycosylated NCs; star, unprocessed NCs.
## TABLE 1

**Differential sensitivity of VCAM1 SP mutants to CAM741**

| VCAM1 (Δ2-10) SP sequence | IC50 (nM) |
|---------------------------|-----------|
| MPGKMVILGASNILWIMFAASQA-FKIE |           |
| n | h | c | mature |
| 11 | 25 | 28 |         |
| Δ2-10; wt | MASNILWIMFAASQA-FKIE | 9.2 |
| Δ2-10; S12G | MASNILWIMFAASQA-FKIE | 43.5 |
| Δ2-10; N13L | MASNILWIMFAASQA-FKIE | 8.2 |
| Δ2-10; I14V | MASNILWIMFAASQA-FKIE | 1.6 |
| Δ2-10; L15V | MASNILWIMFAASQA-FKIE | 5.0 |
| Δ2-10; I14V, L15V | MASNILWIMFAASQA-FKIE | 7.2 |
| Δ2-10; W16F | MASNILWIMFAASQA-FKIE | 12.6 |
| Δ2-10; I17V | MASNILWIMFAASQA-FKIE | 3.1 |
| Δ2-10; I14V, I17V | MASNILWIMFAASQA-FKIE | 4.3 |
| Δ2-10; M18A | MASNILWIMFAASQA-FKIE | 7.1 |
| Δ2-10; W16L | MASNILWIMFAASQA-FKIE | 15.5 |
| Δ2-10; M18I | MASNILWIMFAASQA-FKIE | 32.4 |
| Δ2-10; W16L, M18I | MASNILWIMFAASQA-FKIE | 309.2 |
| Δ2-10; A20V | MASNILWIMFAASQA-FKIE | 23.6 |
| Δ2-10; A20I | MASNILWIMFAASQA-FKIE | 53.6 |
| Δ2-10; A21V | MASNILWIMFAASQA-FKIE | 129.1 |
| Δ2-10; M18I, A21V | MASNILWIMFAASQA-FKIE | 271.9 |
| Δ2-10; A20V, A21V | MASNILWIMFAASQA-FKIE | 600.3 |
| Δ2-10; S22V | MASNILWIMFAASQA-FKIE | 1.8 |
| Δ2-10; S22V, Q23H, AG | MASNILWIMFAAVG-FKIE | <1.3 |
| Δ2-10; F25G | MASNILWIMFAASQA-FKIE | 113.2 |
| Δ2-10; K26E | MASNILWIMFAASQA-FKIE | 14.1 |
| Δ2-10; I27G | MASNILWIMFAASQA-FKIE | 16.0 |

### VCAM1 (FL) sequence

| IC50 (nM) |
|-----------|
| MPGKMVILGASNILWIMFAASQA-FKIE | 28.6 |
| MPGKMVILGASNILWIMFAASQA-FKIE | 316.2 |

### VCAM1 mouse (FL) sequence

| IC50 (nM) |
|-----------|
| MPVKMVAVLGASTVLWILFAVSQA-FKIE | 474.1 |
| MPVKMVAVLGASTVLWILFAVSQA-FKIE | 292.5 |
| MPVKMVAVLGASTVLWILFAVSQA-FKIE | 88.8 |
| MPVKMVAVLGASTVLWILFAVSQA-FKIE | 42.1 |

HEK293 cells were transfected with different VCAM1 SP-SEAP fusion constructs and incubated with increasing concentrations of CAM741. Twenty-four hours post-transfection, supernatants were harvested and analysed for alkaline phosphatase activity. Results shown are IC50 values from at least three independent experiments performed in triplicates. Mutations are indicated by grey boxes, the cleavage site is underlined. The amino-acid residues of the mature region are boxed.
| VCAM1 (Δ2-10) sequence | IC50 (nM) |
|------------------------|-----------|
| 11 MASNILWIMFAASQA-FKIE | 4.9       |
| 25 MASNILWIMFAASQA-FKIE | 16.5      |
| 28 MASNILWIMFAASQA-FKIE | 10.9      |
| Δ2-10; A11C MASNILWIMFAASQA-FKIE | 16.5      |
| Δ2-10; N13C MASNILWIMFAASQA-FKIE | 10.9      |
| Δ2-10; L15C MASNILWIMFAASQA-FKIE | 4.1       |
| Δ2-10; M18C MASNILWICFAASQA-FKIE | 7.1       |
| Δ2-10; A20C MASNILWIMFCASQA-FKIE | 18.1      |
| Δ2-10; S22C MASNILWIMFAASQA-FKIE | 3.6       |
| Δ2-10; A24C MASNILWIMFAASQA-FKIE | 34.7      |
| Δ2-10; K26C MASNILWIMFAASQA-FKIE | 10.2      |
| Δ2-10; M18I, A21V, K26C MASNILWIMFVVSQA-FKIE | 479.5     |
| Δ2-10; A20V, A21V, K26C MASNILWIMFVVVSQA-FKIE | 370.1     |

HEK293 cells were transfected with different SP-SEAP fusion constructs and incubated with increasing concentrations of CAM741. Twenty-four hours post-transfection, supernatants were harvested and analysed for alkaline phosphatase activity. Results shown are IC50 values from at least three independent experiments performed in triplicates. Mutations are indicated by grey boxes, the cleavage site is underlined. The amino-acid residues of the mature region are boxed.
Figure 1

A

\[ \text{VCAM1 (Δ2-10) SP-SEAP} \]
\[ \text{VCAM1 (FL) SP-SEAP} \]

VCAM1 wt

B

High-resolution gel

VCAM1 (Δ2-10)-SP-SEAP

VCAM1 wt

CAM741

Proteinase K

Triton X-100

Endo F

-  -  -  +  +

-  +  -  +  +

-  -  +  -  +

SP  +4  SEAP mature

146

SP  VCAM1 mature

24  158

182

11

MASNILWIMFAASQA-FKIE-SEAP

25  28

MPGKMVLGASNILWIMFAASQA-FKIE-SEAP

182
Figure 1

C

| CAM741 (nM) |
|-------------|
| 1000        |
| 100         |
| 10          |
| 1           |
| -           |

- SEAP wt
- VCAM1 (Δ2-10) SP-SEAP
- VCAM1 (Δ2-10; A21V) SP-SEAP
- VCAM1 (Δ2-10; M18I, A21V) SP-SEAP
- VCAM1 (Δ2-10; A20V, A21V) SP-SEAP
- VCAM1 (FL) SP-SEAP
- mouse VCAM1 (FL) SP-SEAP
- mouse VCAM1 (FL, V21A) SP-SEAP
- mouse VCAM1 (FL, L18M, V21A) SP-SEAP
- VCAM1 (wt)
- VCAM1 (M18I, A21V)

microsomes
- + + + + + -
Figure 2

Figure showing the results of a protein expression experiment. The figure includes a schematic representation of protein expression levels under different conditions. The experiment involves the use of mutants with specific cysteine substitutions to study the effect of CAM741 on protein localization.

Key:
- wt: wild type
- A11C, N13C, L15C, M18C, A20C, S22C, A24C, K26C: specific cysteine substitutions
- SEAP: secreted alkaline phosphatase
- MASNILWIMFAASQA-FKIE-SEAP: SEAP mature sequence
- (Δ2-10) SP: deletion of the first 10 amino acids
- SP+4: Cys substitution at position 4
- IP Sec61β: immunoprecipitation with Sec61β
- CAM741: treatment with CAM741

Legend:
- +: increased expression
- -: decreased expression

The figure illustrates the altered expression patterns of SEAP in various conditions and indicates the relative amounts of protein in the cytosol and lumen with or without CAM741 treatment.
Figure 3

(C26 25 28) SP+4 SEAP mature

19 54

K26C

M18I, A21V, K26C

A20V, A21V, K26C

CAM741 (nM) 1 10 100 1000

IP Sec61β

IP Sec61α

MW [kDa]

- 45
- 30
- 20.1
- 14.3

C26

54

SEAP mature

19

(Δ2-10) SP

+4

54

SEAP mature

19

(Δ2-10) SP

+4

54

SEAP mature

19

Figure 5

A

VCAM1
N-tag VCAM1
IL-6
N-tag IL-6
N-tag VCAM1 FL+4-SEAP

MMNESSTLADSSATQAN- MPGKMVVILGASNILWIMFAASQA-
MNSFSTSAFGPVAFSLGLLVLPA-
MMNESSTLADSSATQAN- MPGKMVVILGASNILWIMFAASQA-
MNSFSTSAFGPVAFSLGLLVLPA-

B

N-tag SP VCAM mature
VCAM 112 aa
- N-tag + N-tag + N-tag
IL-6 67 aa
N-tag SP IL-6 mature
microsomes CAM741 Endo F
- - - + + + - - - - - -

C

N-tag VCAM 112 aa
CAM741 (nM)
microsomes
+ + + + + + + + + + + +
Figure 5

D

**N-tag**

**SP**

**+4 SEAP mature**

**28**

**54**

![Image](image1.png)

E

**N-tag**

**SP**

**VCAM1 mature**

**24**

**33**

**57**

![Image](image2.png)

N-tag VCAM1 (FL) SP -SEAP IC50 = 4.9 nM

N-tag VCAM1 57 aa

N-tag VCAM1 67 aa

microsomes - + + + +

CAM741 - - + - -

Endo F - - - - +
The translocation inhibitor CAM741 interferes with vascular cell adhesion molecule 1 signal peptide insertion at the translocon

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