

# A substrate-specific inhibitor of protein translocation into the endoplasmic reticulum

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**The segregation of secretory and membrane proteins to the mammalian endoplasmic reticulum is mediated by remarkably diverse signal sequences that have little or no homology with each other<sup>1,2</sup>. Despite such sequence diversity, these signals are all recognized and interpreted by a highly conserved protein-conducting channel composed of the Sec61 complex<sup>3,4</sup>. Signal recognition by Sec61 is essential for productive insertion of the nascent polypeptide into the translocation site<sup>5</sup>, channel gating<sup>6</sup> and initiation of transport. Although subtle differences in these steps can be detected between different substrates<sup>7,8</sup>, it is not known whether they can be exploited to modulate protein translocation selectively. Here we describe cotransin, a small molecule that inhibits protein translocation into the endoplasmic reticulum. Cotransin acts in a signal-sequence-discriminatory manner to prevent the stable insertion of select nascent chains into the Sec61 translocation channel. Thus, the range of substrates accommodated by the channel can be specifically and reversibly modulated by a cell-permeable small molecule that alters the interaction between signal sequences and the Sec61 complex.**

A screen for inhibitors of the expression of cell adhesion molecules led to the identification of HUN-7293, a cyclodepsipeptide natural product that potently inhibits vascular cell adhesion molecule 1 (VCAM1) expression in endothelial cells<sup>9</sup>. The mechanism of action and molecular target of HUN-7293 are unknown. However, key structural features of HUN-7293 important for its activity have been defined by Boger and colleagues in a systematic analysis of 40 structural variants<sup>10</sup>. On the basis of this seminal study, we designed and synthesized a simplified analogue of HUN-7293, which we named 'cotransin' (Fig. 1a). We also synthesized 'nor-cotransin', a putatively inactive variant lacking a critical *N*-methyl moiety<sup>10</sup>.

To evaluate the activity of cotransin and nor-cotransin, primary human cells were stimulated under several conditions and characterized for the expression of 26 cell surface and secreted proteins<sup>11</sup>. Cotransin, but not nor-cotransin, inhibited the stimulated expression of only two proteins, VCAM1 and P-selectin (Fig. 1b, c, and Supplementary Fig. S1). Analysis of either total secreted proteins (Fig. 1d) or cellular glycoproteins (Supplementary Fig. S2) from pulse-labelled COS-7 cells revealed a similarly selective effect. Cotransin therefore selectively inhibits the expression of a small subset of secretory and membrane proteins, including VCAM1.

Selective, potent and reversible inhibition of VCAM1 (Fig. 2a, b), but not other proteins (for example green fluorescent protein; data not shown) expressed heterologously from the constitutive cytomegalovirus promoter, indicated that the site of cotransin action is likely to be post-transcriptional and, possibly, post-translational. Indeed, proteasome inhibition during treatment with cotransin stabilized a non-glycosylated form of VCAM1 (Fig. 2a). This indicated that cotransin might cause newly synthesized VCAM1 to be degraded

rather than productively enter the secretory pathway, a conclusion consistent with data reported in a recent patent application<sup>12</sup>.

To identify the step at which VCAM1 is rerouted from a biosynthetic to a degradative fate, its translation and translocation were analysed *in vitro* by using reticulocyte lysate containing rough endoplasmic reticulum (ER) microsomes (RM). Whereas the translocation of VCAM1 was unaffected by cotransin, its translocation into RM was markedly inhibited (Fig. 2c). This is evident by a decrease in VCAM1 glycosylation and complete digestion of non-glycosylated product by exogenous protease (Fig. 2c, lanes 6 and 7). By contrast, translocation reactions containing nor-cotransin (or dimethylsulphoxide; DMSO) resulted in efficient glycosylation and protease protection of VCAM1 (Fig. 2c, lanes 3, 4, 8 and 9). Both VCAM1 translocation and its inhibition by cotransin were observed even when glycosylation was prevented (Fig. 2d), whereas the addition of cotransin after translocation had no effect on either glycosylation or protease protection (data not shown). In striking contrast to VCAM1, the translocation of pre-prolactin (pPrl) was unaffected by cotransin (Fig. 2c, d). Moreover, an analysis of model type I, type II and multi-spanning membrane proteins with cotransin showed no effect on their proper translocation, glycosylation, membrane insertion or topology *in vitro* (Supplementary Fig. S3). These data suggest that cotransin inhibits VCAM1 expression in cells by selectively preventing its cotranslational translocation into the ER.

The amino-terminal signal sequences that mediate the translocation of VCAM1 and pPrl into the ER are markedly different from one another (Fig. 2e). To test whether this difference underlies cotransin's substrate selectivity, we prepared chimaeric constructs in which the signals of VCAM1 and pPrl were fused to an otherwise non-translocated reporter. Although both signals directed efficient translocation of the reporter, only the VCAM1 fusion construct was inhibited by cotransin (Fig. 2e). Identical results were obtained when another reporter (the prion protein) was fused to the VCAM1 and pPrl signals (data not shown). Sensitivity to cotransin is therefore determined primarily by the signal sequence. Although these results indicate that the VCAM1 signal sequence could be the direct target of cotransin, examination of additional cotransin-sensitive and cotransin-resistant substrates indicated that this is improbable (Supplementary Table 1). Comparison of their signal sequences failed to reveal either a consensus cotransin-binding motif shared by the sensitive substrates or the basis for cotransin resistance. We therefore focused on the targeting and translocation machinery, components of which are known to interact with diverse signal sequences, as potential targets of cotransin.

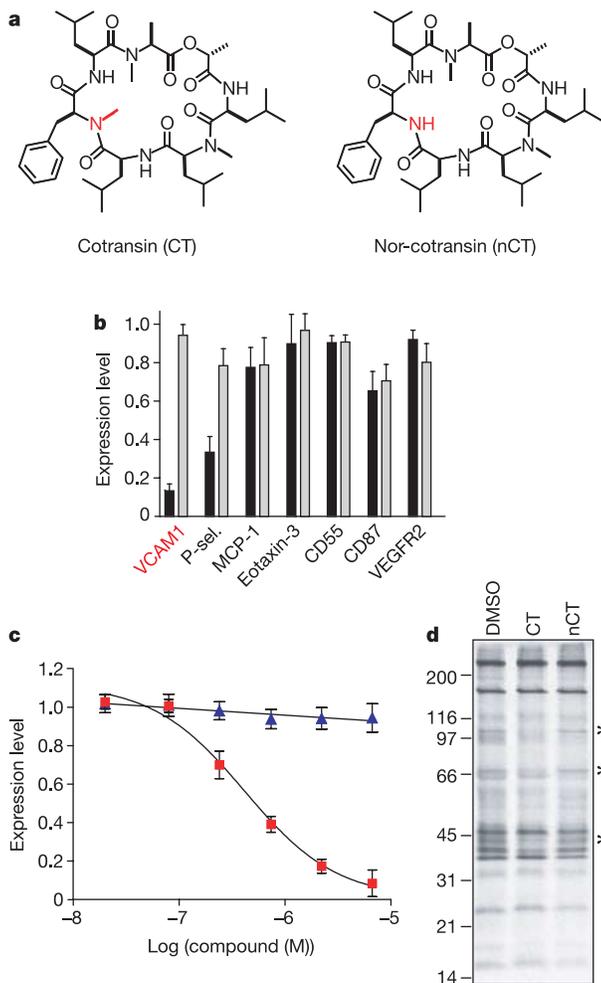
To identify the step of protein translocation inhibited by cotransin, we analysed interactions between VCAM1 nascent chains and the translocation machinery by chemical crosslinking. In the cytosol, 145-residue ribosome-nascent chain complexes (RNCs) of VCAM1 formed crosslinks with several proteins, none of which were affected

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by cotransin (Fig. 3a, lanes 7–9). This pattern of crosslinks changed when the RNCs were prepared in the presence of RM, indicating a clear shift in the environment surrounding the nascent chain (Fig. 3a, lanes 10–12). As expected, a major cytosolic crosslink was identified by immunoprecipitation as SRP54 (Fig. 3a, lanes 7–9), the signal sequence-binding subunit of SRP<sup>13,14</sup>. This interaction was unper-  
turbed by cotransin (Fig. 3a, lane 8) but was lost after the addition of microsomes (lanes 10–12). Taken together, the identical crosslinking patterns before targeting, equal efficiencies of SRP54 interaction and equal efficiencies of SRP release are evidence that cotransin has no effect on the steps preceding the SRP receptor (SR)-mediated transfer of RNCs to the translocation channel. These results also argue against the VCAM1 signal sequence itself as the direct target of cotransin.

Instead, the first cotransin-sensitive step occurs at the membrane.

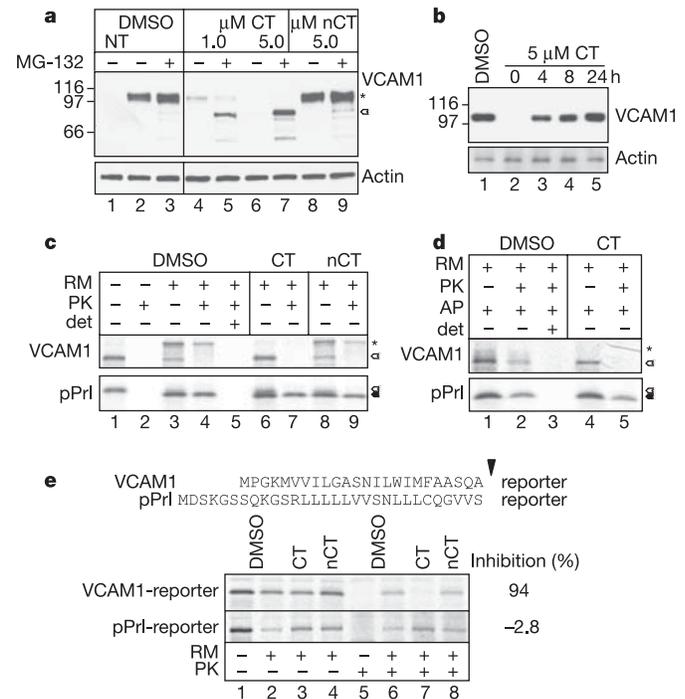


**Figure 1 | Cotransin inhibits expression of a subset of secreted and membrane proteins. a**, Structures of cotransin and nor-cotransin.

**b**, Primary human endothelial cells were treated with DMSO, 2  $\mu$ M cotransin (black bars) or 2  $\mu$ M nor-cotransin (grey bars) and stimulated with 5 ng ml<sup>-1</sup> interleukin-4 and 10  $\mu$ M histamine. After 24 h, cell surface proteins were quantified by enzyme-linked immunosorbent assay. Protein expression levels were normalized to DMSO controls (means  $\pm$  s.e.m.;  $n = 6$ ). Additional results are shown in Supplementary Fig. S1. MCP-1, monocyte chemoattractant protein-1; P-sel, P-selectin; VEGFR2, vascular endothelial growth factor receptor-2. **c**, Dose-response curve showing the effect of cotransin (red squares) and nor-cotransin (blue triangles) on VCAM1 expression (means  $\pm$  s.e.m.;  $n = 6$ ). The IC<sub>50</sub> is about 0.5  $\mu$ M, compared with about 1 nM for HUN-7293 (refs 9, 10). **d**, Effect of cotransin (5  $\mu$ M) on total secreted proteins from pulse-labelled COS-7 cells. Asterisks indicate secreted proteins whose levels are reduced by cotransin. Molecular masses are in kDa.

Signal sequence cleavage, an indicator of nascent chain access to signal peptidase on the luminal side of the membrane, was not observed with cotransin (Fig. 3a, lanes 5 and 11). Furthermore, cotransin, but not nor-cotransin, significantly altered the pattern of crosslinks between RNCs and ER proteins (Fig. 3a, lanes 10–12; Fig. 3b, lanes 4–6). Differences in crosslinking were apparent with RNCs of several different lengths and with both lysine-reactive and cysteine-reactive crosslinkers (Fig. 3a, b, and data not shown). Immunoprecipitation studies identified the primary differences (Fig. 3b). RNCs in DMSO and nor-cotransin samples crosslinked efficiently to Sec61 $\alpha$  and luminal chaperones (such as protein disulphide isomerase; PDI), but not to Sec61 $\beta$ . By contrast, RNCs synthesized in the presence of cotransin showed diminished Sec61 $\alpha$  and PDI crosslinks but enhanced Sec61 $\beta$  crosslinks. RNCs are therefore in close proximity to the Sec61 complex with or without cotransin; however, their orientation with respect to Sec61 subunits is significantly perturbed by cotransin.

Sedimentation, protease protection and transport assays of translocation intermediates led to similar conclusions. In these experiments, RNCs in the cotransin, nor-cotransin and DMSO



**Figure 2 | Cotransin inhibits cotranslational translocation. a**, COS-7 cells were transfected with a VCAM1 expression plasmid or not transfected (NT) and treated with cotransin (CT) or nor-cotransin (nCT). The proteasome inhibitor MG-132 (5  $\mu$ M) was included where indicated. After 24 h, cell lysates were analysed by immunoblotting with anti-VCAM1 or anti-actin antibodies. **b**, Cells were transfected as in **a**, except that cotransin was removed from the medium after 24 h. Cells were harvested at specified time points and analysed for VCAM1 expression. Molecular masses in **a** and **b** are in kDa. **c**, Transcripts encoding VCAM1 or pre-prolactin (pPrI) were translated in the presence of [<sup>35</sup>S]methionine and, where indicated, rough ER microsomes (RM). Translation reactions also contained 10  $\mu$ M cotransin, 10  $\mu$ M nor-cotransin or DMSO. Equal aliquots of the translated material were left untreated or treated with PK in the presence or absence of Triton X-100 (det). Samples were separated by SDS-PAGE and analysed by autoradiography. The positions of precursor (open arrow), signal-cleaved (solid arrow) and glycosylated (asterisk) species are indicated. **d**, As in **c**, except that a peptide inhibitor of glycosylation (AP) was included in the translation reactions. **e**, VCAM1 and pPrI signal sequences (shown) were appended to the N terminus of a Gal4-NF $\kappa$ B fusion protein ('reporter') and the resulting constructs were tested in cell-free translocation assays as in **c** (quantified by phosphorimager analysis).

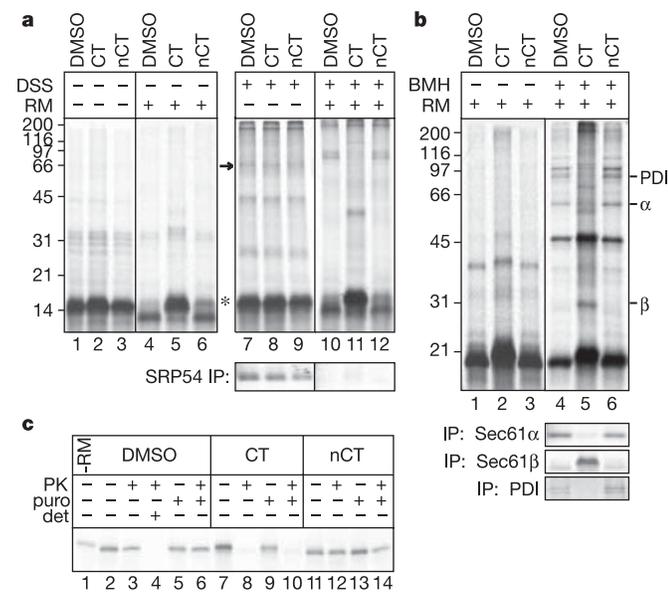
samples quantitatively achieved salt-resistant binding to microsomes, a previously defined indicator of tight interaction between ribosome and translocon after transfer from SRP<sup>5,15,16</sup>. Treatment of these salt-resistant translocation intermediates with protease showed that cotransin-treated RNCs were accessible to exogenous protease (Fig. 3c, lane 8), whereas the control samples were protease-protected in the absence, but not the presence, of detergent (lanes 3, 4 and 12). The cotransin-treated RNCs remained accessible to protease even after release from the ribosome with puromycin (Fig. 3c, lane 10), indicating that although they were at the translocon, they could not translocate into the lumen. Protease accessibility and inhibition of translocation were observed only if cotransin was present during the assembly of translocation intermediates. Addition after translocation intermediate assembly had no effect (data not shown), indicating that once nascent chains have initiated translocation, they are no longer influenced by cotransin. Cotransin therefore acts at a step after the targeting and transfer of RNCs to the translocon, but before the nascent chains have access to the ER lumen.

The post-targeting step inhibited by cotransin involves insertion of the nascent chain into the translocation channel and opening of the channel towards the lumen, and proteins engaged in this step are potential molecular targets of cotransin. These include the Sec61 complex<sup>5</sup> and several accessory components of the translocon (such as TRAM<sup>15,17</sup>, the TRAP complex<sup>18</sup>, the Sec62/63 complex<sup>19,20</sup> or BiP<sup>20–23</sup>). However, removal of these and other components from the membrane or lumen had little effect on VCAM1 translocation beyond that observed for pPrI (Supplementary Figs S4 and S5). These accessory components are therefore largely dispensable for

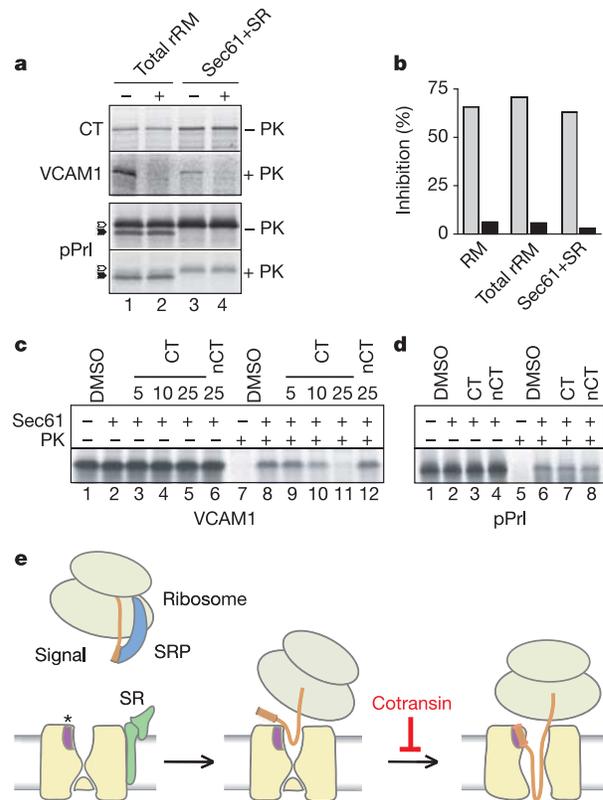
VCAM1 translocation and are unlikely to be targets for cotransin inhibition. The only protein whose depletion prevented VCAM1 translocation was the Sec61 complex (Supplementary Fig. S5), a component also essential for pPrI translocation<sup>24</sup>.

Because every known accessory protein previously implicated in translocation was represented in these depletions, the target of cotransin is likely to be a novel protein or the Sec61 complex itself. To explore the latter possibility, we prepared proteoliposomes containing only the Sec61 complex and SR (Supplementary Fig. S6), the minimal machinery required for protein translocation<sup>24</sup>. VCAM1 and pPrI were both translocated into the lumen of these minimal proteoliposomes (at reduced efficiency); however, only VCAM1 was inhibited by cotransin (Fig. 4a, b). Given that targeting to the translocon is unaffected by cotransin (Fig. 3a), these results suggest that cotransin acts to prevent signal sequence-dependent gating of the Sec61 translocation channel.

To test this hypothesis directly, we examined the interaction between VCAM1 or pPrI nascent chains and purified Sec61. A



**Figure 3 | Cotransin inhibits insertion of VCAM1 into the translocation channel.** **a**, VCAM1 nascent chains of 145 residues (145-mers) were synthesized in the absence or presence of RM, isolated by centrifugation and subjected to crosslinking with disuccinimidyl suberate (DSS). Samples were separated by SDS-PAGE and analysed by autoradiography. Crosslinks to SRP54 (arrow, lanes 7–9) were confirmed by immunoprecipitation (IP). The position of non-signal-cleaved VCAM1 is indicated with an asterisk. **b**, VCAM1 180-mers were translated in the presence of RM, isolated by centrifugation through a high-salt cushion, and subjected to crosslinking with bismaleimido-hexane (BMH). Crosslinks to Sec61 $\alpha$ , Sec61 $\beta$  and PDI (marked by arrows) were confirmed by immunoprecipitation. Molecular masses in **a** and **b** are in kDa. **c**, VCAM1 180-mers were translated in the presence of RM and isolated as in **b**. Equal aliquots were treated with puromycin (puro) and PK as indicated. Cotransin or non-cotransin was included during the translation reaction at a final concentration of 25  $\mu$ M. Det, detergent (Triton X-100).



**Figure 4 | Cotransin prevents signal sequence recognition by the Sec61 complex.** **a**, Full-length VCAM1 or pPrI was translated in the presence of proteoliposomes containing total ER membrane proteins (total rRM) or purified Sec61 complex and SRP receptor (Sec61/SR). Where indicated, cotransin (10  $\mu$ M) was included. **b**, Translocation data from panel **a** were quantified by phosphorimager analysis. Grey bars, VCAM1; black bars, pPrI. **c**, VCAM1 145-mer RNCs were isolated by centrifugation through a high-salt cushion. RNCs were incubated with purified Sec61 complex in the presence of compounds as indicated. Samples were left untreated or treated with PK. Concentrations of cotransin and non-cotransin are shown in  $\mu$ M. **d**, As in **c**, except that pPrI 145-mers were used. Cotransin and non-cotransin were used at a final concentration of 25  $\mu$ M. **e**, Model of cotransin activity. SRP-mediated targeting of the RNC to a Sec61-containing translocon in the ER membrane is unaffected by cotransin. Rather, cotransin blocks signal sequence-dependent insertion of the nascent chain into the translocation channel and simultaneous opening of the pore towards the lumen of the endoplasmic reticulum.

productive interaction between RNCs and Sec61 shields the nascent chain from protease digestion because of its insertion into the translocation channel<sup>5,25</sup>. Using this assay, we found that purified 145-mer RNCs of either VCAM1 or pPrl, which are readily digested by protease, become protected on addition of the Sec61 complex (Fig. 4c, compare lanes 7 and 8; Fig. 4d, compare lanes 5 and 6). Remarkably, insertion of the VCAM1 nascent chain into the Sec61 channel is selectively inhibited by cotransin (Fig. 4c, lanes 11 and 12). Cotransin had no effect on the pPrl nascent chain (Fig. 4d). Thus, cotransin acts in a signal sequence-discriminatory manner to prevent the nascent chain from interacting productively with the Sec61 channel (Fig. 4e).

In this study we have characterized cotransin, the first small-molecule modulator of protein translocation into the ER. Cotransin inhibits translocation in a signal sequence-dependent manner, despite targeting a translocation channel utilized by all secretory and membrane proteins. Identification of the exact features in the signal that confer sensitivity (and resistance) to cotransin must await systematic mutagenesis studies. However, it is already apparent that differences between natural signal sequences can markedly influence sensitivity to cotransin (Supplementary Table 1). This implies that signal sequence variation can be exploited to modulate the functional expression of secreted and membrane proteins at their point of entry into the secretory pathway.

The precise molecular mechanism by which cotransin inhibits nascent chain insertion and gating of the Sec61 channel for select substrates is not known. One possibility is that cotransin stabilizes the channel in a closed conformation such that gating by low-affinity signal sequences is inefficient. Alternatively, cotransin might directly or allosterically alter the topography of a signal sequence binding site, postulated to involve transmembrane helices 2 and 7 of Sec61 $\alpha$ <sup>26,27</sup>. In this model, the altered binding site would be less accommodating or flexible, resulting in increased substrate selectivity. Deciphering the molecular details of how cotransin influences the signal sequence interaction with the Sec61 channel is likely to shed light on the mechanism by which unrelated signal sequences gate a communal translocation channel. Cotransin thus provides a powerful new probe for investigating the molecular function of a protein-conducting channel as well as a selective, reversible method of inhibiting cotranslational translocation in living cells.

## METHODS

**Constructs.** The coding region for human VCAM1 was amplified by polymerase chain reaction with reverse transcription (RT-PCR) from total RNA isolated from tumour necrosis factor- $\alpha$ -stimulated A549 cells (gift from H. Luecke) and cloned into the pcDNA3.1 vector (Invitrogen). Bovine pPrl in the SP64 vector (Promega) has been described previously<sup>18</sup>. The Gal4-NF $\kappa$ B coding region (from plasmid pBD-NF $\kappa$ B (Stratagene)) containing a carboxy-terminal KDEL sequence was amplified by PCR and subcloned into signal sequence cassettes<sup>8</sup> containing the VCAM1 or pPrl signal sequence to generate the signal-Gal4-NF $\kappa$ B constructs. All constructs were verified by sequencing.

**Antibodies and proteins.** Antibodies against Sec61 $\beta$  were described previously<sup>18</sup>. Antibodies against Sec61 $\alpha$  were a gift from R. Gilmore. Antibodies against VCAM1, PDI, actin and SRP54 were purchased from Santa Cruz, Stressgen, Sigma and BD Biosciences, respectively. Mammalian Sec61 complex and SR were purified from canine RM as described previously<sup>24</sup>. Immunoblotting relative to a titration of starting RM at a defined concentration of 1 equivalent (equiv)  $\mu$ l<sup>-1</sup> was used to determine the concentration of purified proteins. Abundances of about 2 pmol equiv<sup>-1</sup> and about 0.2 pmol equiv<sup>-1</sup> for the Sec61 complex and SR, respectively, have been established in previous studies<sup>24</sup>.

**Small molecule synthesis.** Cotransin and nor-cotransin were synthesized by adapting the published synthesis of HUN-7293 (ref. 28) and were characterized by <sup>1</sup>H-NMR and mass spectrometry.

**Mammalian cell culture and transient expression of VCAM1.** A detailed description of cell-based models of inflammation is given in Supplementary Methods. COS-7 cells were maintained in accordance with standard procedures. Pulse-labelling of secreted proteins was performed on cells preincubated for 60 min at 37 °C with cotransin or nor-cotransin (5  $\mu$ M) in medium lacking

methionine, cysteine and serum. After being labelled with 400  $\mu$ Ci ml<sup>-1</sup> [<sup>35</sup>S]methionine/cysteine for 30 min, proteins in the medium were collected by precipitation with 15% trichloroacetic acid, washed in acetone, dissolved in 1% SDS, 0.1 M Tris-HCl pH 8, and analysed by SDS-polyacrylamide-gel electrophoresis (SDS-PAGE) and autoradiography. VCAM1 expression analysis was performed 24 h after transfection with Lipofectamine 2000 (Invitrogen). Where indicated, cotransin, nor-cotransin or MG-132 (5  $\mu$ M; Calbiochem) was added 5 h after transfection. Cells were harvested in 1% SDS, 0.1 M Tris-HCl pH 8 and analysed by SDS-PAGE and immunoblotting.

**Cell-free translocation assays.** *In vitro* transcription, translation, translocation, and protease protection assays were performed as described previously<sup>8,15</sup>. Truncated transcripts lacking a stop codon were synthesized from PCR-generated templates<sup>5</sup>. Translations were at 32 °C for 60 min (full-length constructs) or for 20 min (truncated constructs). Nascent chains were isolated at 4 °C by sedimentation in a TLA 100.3 rotor (75,000 r.p.m. for 45 min (for RNCs) or 70,000 r.p.m. for 10 min (for membrane-bound RNCs)) through a sucrose cushion (100  $\mu$ l, 0.5 M) in PSB buffer (50 mM HEPES pH 7.4, 150 mM potassium acetate, 5 mM magnesium acetate). Translation reactions to measure salt-resistant binding were adjusted to 0.5 M potassium acetate and sedimented through a 0.5 M sucrose cushion containing 0.5 M potassium acetate in PSB. Isolated nascent chains were resuspended in PSB containing 0.25 M sucrose before further manipulations. Treatment with puromycin (1 mM; Calbiochem) was for 15 min at 25 °C (Fig. 3c). In Fig. 4c, d, nascent chains were isolated in the absence of compound, then resuspended in the presence of cotransin or nor-cotransin (25  $\mu$ M) before treatment with proteinase K (PK). Inhibition of glycosylation was with a competitive peptide inhibitor (NH<sub>2</sub>-Asp-Tyr-Thr-COOH; California Peptide Research) at 100  $\mu$ M (Fig. 2d). Quantitative analysis of translocation experiments used a Typhoon 9400 phosphorimager (Amersham) with accompanying software.

**Crosslinking assays.** Crosslinking with 145-mers was with 0.5 mM disuccinimidyl suberate (Pierce) at 23 °C for 30 min; the reaction was quenched with 0.1 M Tris-HCl pH 8.0, 1% saponin (Sigma), 10 mM EDTA, 50  $\mu$ g ml<sup>-1</sup> RNase A (Sigma), then added to 1% SDS, 0.1 M Tris-HCl pH 8 and heated to 37 °C. Crosslinking with 180-mers (isolated in the presence of high salt concentration) was with 50  $\mu$ M bismaleimido-hexane (Pierce) at 23 °C for 15 min; the reaction was quenched with 100 mM 2-mercaptoethanol, then added to 1% SDS, 0.1 M Tris-HCl pH 8 and heated to 37 °C. Immunoprecipitation with the indicated antibodies was performed after the dilution of samples tenfold with 1% Triton X-100, 50 mM HEPES pH 7.4, 100 mM sodium chloride.

**Reconstituted proteoliposomes.** Glycoprotein-depleted and Q Sepharose-depleted proteoliposomes were prepared as described previously<sup>18</sup>. Sec61-depleted proteoliposomes and proteoliposomes containing purified Sec61 and SR were prepared as described<sup>24</sup>.

**Assays with RNCs and purified Sec61 complex in detergent solution.** Nascent 145-mer chains translated in the absence of compound and isolated in the presence of high salt concentration were resuspended in half the original volume of 50 mM HEPES pH 7.4, 25 mM potassium acetate, 2 mM magnesium acetate, 0.3% DeoxyBigCHAP (Calbiochem) and 25  $\mu$ M cotransin or nor-cotransin where indicated. Purified Sec61 complex was added to a final concentration of about 500 nM and the samples were incubated at 32 °C for 20 min before transfer to ice for protease protection assays. Proteolysis reactions were with 0.5 mg ml<sup>-1</sup> PK for 60 min on ice as described<sup>18</sup>.

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