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Control of glycosylation of MHC class II-associated invariant chain by translocon-associated RAMP4

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Protein translocation across the membrane of the endoplasmic reticulum (ER) proceeds through a proteinaceous translocation machinery, the translocon. To identify components that may regulate translocation by interacting with nascent polypeptides in the translocon, we used site-specific photo-crosslinking. We found that a region C-terminal of the two N-glycosylation sites of the MHC class II-associated invariant chain (Ii) interacts specifically with the ribosome-associated membrane protein 4 (RAMP4). RAMP4 is a small, tail-anchored protein of 66 amino acid residues that is homologous to the yeast YSY6 protein. YSY6 suppresses a secretion defect of a *secY* mutant in *Escherichia coli*. The interaction of RAMP4 with Ii occurred when nascent Ii chains reached a length of 170 amino acid residues and persisted until Ii chain completion, suggesting translocational pausing. Site-directed mutagenesis revealed that the region of Ii interacting with RAMP4 contains essential hydrophobic amino acid residues. Exchange of these residues for serines led to a reduced interaction with RAMP4 and inefficient N-glycosylation. We propose that RAMP4 controls modification of Ii and possibly also of other secretory and membrane proteins containing specific RAMP4-interacting sequences. Efficient or variable glycosylation of Ii may contribute to its capacity to modulate antigen presentation by MHC class II molecules.

Keywords: endoplasmic reticulum/glycosylation/invariant chain/protein translocation/RAMP4

Introduction

Cotranslational translocation of proteins across the membrane of the endoplasmic reticulum (ER) involves signal recognition particle (SRP), SRP receptor (docking protein), a translocation pore (translocon) and several accessory proteins involved in co- or post-translational modification (reviewed by Walter and Johnson, 1994; Lütcke, 1995; Rapoport *et al.*, 1996).

Passage of the nascent polypeptide chain across the membrane occurs through an aqueous pore in the translocon (for review see Rapoport *et al.*, 1996; Johnson, 1997). The core component of the translocon is the Sec61p complex which consists of three subunits (α, β and γ) (Görlich and Rapoport, 1993; Beckmann *et al.*, 1997; Matlack *et al.*, 1998). Besides the core components, other proteins have been shown to participate in translocation or are associated with membrane-bound ribosomes during protein translocation. The TRAM protein was shown to function at an early step of membrane insertion of some proteins (Görlich and Rapoport, 1993; Voigt *et al.*, 1996). Proteins of the translocation site with unknown function are the ribosome-associated membrane protein 4 (RAMP4) and the TRAP complex (Rapoport *et al.*, 1996).

During their translocation across the membrane the nascent polypeptides are often modified. Signal sequences are cleaved by signal peptidase, a protein complex consisting of five different subunits which expose the catalytic site to the ER lumen (Evans *et al.*, 1986; Shelness, 1993). Oligosaccharide moieties can be bound to asparagine residues of a consensus site for N-glycosylation, asparagine (N)-X-threonine (T) / serine (S) (Abeijon and Hirschberg, 1992; Kelleher *et al.*, 1992). This glycosylation is mediated by oligosaccharyl transferase (OST), which transfers an oligosaccharyl moiety from a dolichol-carrier to the asparagine residue. Other protein modifications are the formation of disulfide bridges by protein disulfide isomerases (Hasel *et al.*, 1991) and the addition of lipids to nascent polypeptides (Gordon *et al.*, 1995).

Proteins that come into contact with nascent polypeptide chains during their translocation across the ER membrane have been identified by crosslinking (Krieg *et al.*, 1989; Wiedmann *et al.*, 1989; Görlich *et al.*, 1991). Chemical crosslinking methods as well as site-specific photo-crosslinking methods have been employed in these studies (Görlich *et al.*, 1991; Brunner, 1993; Martoglio and Dobberstein, 1996). One type of site-specific crosslinking makes use of suppressor tRNA to incorporate the UV-activatable crosslinking reagent (Tmd)Phe into nascent polypeptides (High *et al.*, 1993b). When activated, the resulting carbene radical reacts within nanoseconds with neighbouring molecules independently of their chemical nature (Brunner, 1989). Employing this method, signal and signal anchor sequences have been found to contact

proteins and lipids during their membrane insertion (High *et al.*, 1993b; Mothes *et al.*, 1994; Martoglio *et al.*, 1995). The translocating portion of a nascent polypeptide chain has been shown to be in contact with Sec61α and/or TRAMp during various stages of translocation (High *et al.*, 1993b; Mothes *et al.*, 1994). Crosslinking efficiencies to these components were low, suggesting quenching by water (Martoglio *et al.*, 1995). This is consistent with an aqueous channel demonstrated directly by fluorescent probes (Crowley *et al.*, 1994) and differential extraction with chaotropic agents (Gilmore and Blobel, 1985). Besides the components of the Sec61p complex and TRAMp, other as yet unidentified proteins were found to contact translocating polypeptides (Mothes *et al.*, 1994; Hegde and Lingappa, 1997; Laird and High, 1997).

Protein translocation across the ER membrane does not always proceed continuously but can pause at distinct sites (Chuck and Lingappa, 1992; Nakahara *et al.*, 1994). Apolipoprotein B (apo B), for example, was shown to contain several pause transfer sequences that pause translocation at many sites during its translocation (Chuck and Lingappa, 1992). Pausing of apo B resulted in opening of the ribosome membrane junction and exposure of parts of nascent apo B to the cytoplasm (Hegde and Lingappa, 1996). Pause transfer sequences from apo B have been identified. They are degenerate and contain characteristic positively charged and hydroxylated amino acid residues (Chuck and Lingappa, 1992, 1993). For one pause transfer sequence an interaction with an 11 kDa membrane protein was found. This protein was probably neither Sec61β nor RAMP4 (Hegde and Lingappa, 1996). Translocational pausing was proposed to be a general mechanism by which a nascent polypeptide is segregated during translocation and subjected to efficient cotranslational modifications (Hegde and Lingappa, 1996).

For molecules involved in antigen presentation, such as the MHC class II molecules, correct folding, modification and oligomeric assembly are particularly important. Changes in these molecules may lead to differences in peptides presented to T cells. MHC class II molecules assemble in the ER into nonameric complexes with invariant chains (Ii) (Pieters, 1997). Ii directs the complex to endosomes where it is proteolytically processed and only a small peptide of Ii, called CLIP, remains bound (Cresswell, 1996). CLIP peptides are exchanged for antigenic peptides which are then presented by MHC class II molecules to T cells (Denzin and Cresswell, 1995; Kropshofer *et al.*, 1998).

To investigate membrane insertion of Ii and the regulation of this process, we identified components that interact with Ii during different stages of membrane insertion. We found that a region C-terminal to the second glycosylation site of Ii interacts with a small protein that we identified as RAMP4. Mutated Ii that is unable to interact with RAMP4 became inefficiently glycosylated. We propose that RAMP4 is a regulatory determinant of protein modification at the translocon.

Results

Identification of components interacting with a translocation intermediate of Ii

To study membrane insertion and translocation of the type II membrane protein invariant chain (Ii) we used an *in vitro* protein synthesizing system supplemented with pancreatic rough microsomes. Combined with site-specific photo-crosslinking, components can be identified that contact nascent chains during their insertion into the membrane (Martoglio and Dobberstein, 1996). We used this approach to identify components that interact with translocating portions of nascent Ii. Ii is a type II membrane protein of 216 amino acids in length. It spans the membrane close to the N-terminus and exposes the C-terminal 156 amino acids to the exoplasmic space. Ii contains two consensus sites for N-glycosylation [asparagine (N)-X-threonine (T) / serine (S)] at positions 114 and 120 (Figure 1A). To identify components interacting with the region near the second N-glycosylation consensus site, we incorporated the amino acid analogue (Tmd)Phe, which contains a crosslinking function at positions 119, 121, 123 and 126 of Ii (Figure 1A). Incorporation of (Tmd)Phe (F*) was achieved by introducing amber stop codons in the mRNA coding for Ii and the use of an amber suppressor tRNA chemically amino-acylated with (Tmd)Phe. Ii mRNAs, truncated in the coding region, were used in an *in vitro* translation–translocation system to generate membrane-inserted Ii translocation intermediates that were still attached to ribosomes (Figure 1B). Such Ii chains were inserted into the membrane in a loop-like configuration with the N-terminus in the cytosol, the middle portion in the ER lumen and the C-terminus attached to the ribosome (Figure 1B).

Truncated mRNA, yielding Ii of 187 amino acids in length, was translated *in vitro* in the presence of pancreatic microsomal membranes and translocation intermediates of each of the respective IiF* mutants synthesized. One half of each sample was subjected to UV light for crosslinking. Proteins were then separated by SDS–PAGE and ^{35}S-labelled proteins visualized by phosphorimaging. Crosslinked products were identified by their reduced electrophoretic mobility compared with the non-crosslinked Ii chains. Several translation products can be distinguished in Figure 1C: 'Iistop' is the Ii fragment that is released at the introduced amber stop codon and 'Iistop g' is its glycosylated form. Successful suppression yielded IiF* translocation intermediates of 187 amino acids in length 'IiF*/187' and its glycosylated form 'IiF*/187g'. In two samples glycosylation was prevented by the addition of a glycosylation acceptor tripeptide (Figure 1C, lanes 7–10). Samples exposed to UV light show crosslinked products for the glycosylated and unglycosylated IiF*^{123}/187 protein (Figure 1C, cf. lanes 5, 6 and 7, 8). The crosslinked product is ~7 kDa larger than the IiF*^{123}/187g (Figure 1C, lane 6) or IiF*^{123}/187 (Figure 1C, lane 8) protein. The efficiency of crosslinking to the small component is high: ~35% of IiF*^{123}/187 or IiF*^{123}/187g were crosslinked to the 7 kDa component. Ii F*/187 with (Tmd)Phe at position 119 or 121 yielded no crosslinked products (Figure 1C, cf. lanes 1, 2 and 3, 4).

To identify more closely the region of Ii that interacts with the 7 kDa component, we introduced a stop codon for site-specific crosslinking at position 126 of Ii (Figure 1A). As can be seen in Figure 1C (lanes 9 and 10), crosslinking to the 7 kDa component is also obtained from this position. This indicates that the 7 kDa component interacts with a region C-terminal of the two Ii glycosylation sites.

To test whether crosslinking to the 7 kDa component can still occur when the nascent IiF*^{123}/187 chain is released from the ribosome, we treated the samples with EDTA or puromycin and then induced crosslinking. EDTA chelates Mg^{2+} ions and thereby leads to dissociation of the ribosomal subunits and release of nascent chains. In the presence of puromycin, the nascent chain is released as puromycyl-peptide. As can be seen in Figure 2, the release of the IiF*^{123}/187 chain from the ribosome by either treatment abolished crosslinking to the 7 kDa component (Figure 2, cf. lane 2 with lanes 4 and 6). This indicates that the interaction of Ii with the small component requires the nascent chain to be attached to the ribosome.

Candidate proteins for the 7 kDa crosslinked component

Two of the known ribosome and translocon-associated proteins, Sec61γ and RAMP4, have molecular masses of ~7 kDa (Rapoport *et al.*, 1996). While the amino acid sequence of Sec 61γ is known and antibodies are available (Hartmann *et al.*, 1994), the sequence of RAMP4 was not yet known. We have purified RAMP4 and determined its N-terminal sequence. Degenerate oligonucleotides were synthesized and complementary cDNA clones from a rat cDNA library isolated and sequenced. The DNA sequence has been submitted to the DDBJ/EMBL/GenBank data library (accession numbers: AF100470 and AJ238236). The deduced amino acid sequence and the sequence obtained from the N-terminal end of RAMP4 are shown in Figure 3. RAMP4 is a protein of 66 amino acid residues with a rather hydrophilic N-terminal half and a stretch of hydrophobic amino acid residues in the C-terminal half (Figure 3, black bar). Searching protein data libraries with the rat RAMP4 sequence revealed homologous sequences from several species including yeast (Figure 3). The yeast homologue, YSY6, has been shown to suppress a defect in protein translocation of a *secY* mutant in *Escherichia coli* (Sakaguchi *et al.*, 1991).

Identification of the 7 kDa component

In order to identify the 7 kDa protein that was crosslinked to IiF*^{123}/187 chains, we employed antisera against Sec61γ and RAMP4. The antisera were raised against peptides representing the N-terminal hydrophilic domains of the two proteins. Antibodies directed against the N-terminal 34 amino acid residues of RAMP4 precipitated the IiF*^{123}/187 crosslinked product (Figure 4, lane 4). The corresponding RAMP4 peptide competed in the immunoprecipitation for the crosslinked product (Figure 4, lane 5). An antiserum directed against Sec61γ did not immunoprecipitate the crosslinked product (Figure 4, lane 6). These results indicate that the protein crosslinked to IiF*^{123}/187 is RAMP4. The component crosslinked to IiF*^{126}/187 was also identified as RAMP4 (data not shown). We conclude that a sequence located C-terminal of the two Ii N-glycosylation sites interacts during translocation with RAMP4.

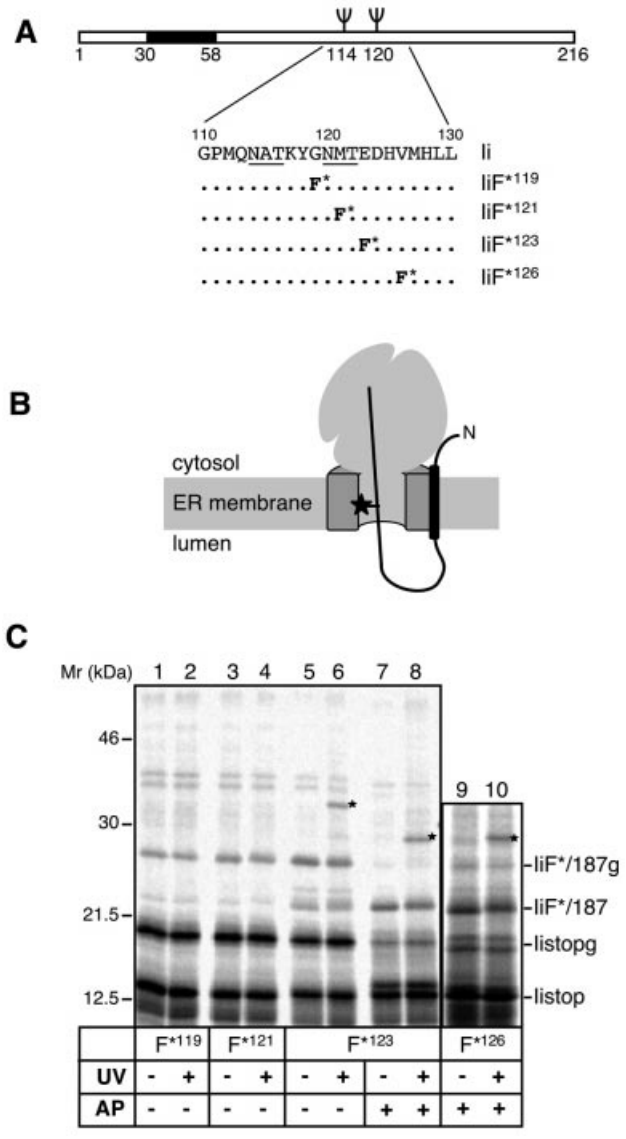

Fig. 1. Site-specific crosslinking of nascent Ii to components of the translocon. (**A**) Outline of Ii and mutants of Ii with the site-specific incorporated photo-activatable amino acid analogue (Tmd)Phe (F*). The linear structure of the type II membrane protein Ii is shown. The black box indicates the signal anchor sequence, forked structures the sites of N-glycosylation at positions 114 and 120. Ii and IiF* mutant sequences between amino acids 110 and 130 are shown. Single amber stop codons for site-specific biosynthetic incorporation of (Tmd)Phe (F*) were placed at residue 119 (IiF*^{119}), 121 (IiF*^{121}), 123 (IiF*^{123}) or 126 (IiF*^{126}). (**B**) Schematic representation of a translocation intermediate of Ii in the ER membrane. The ribosome is attached to the translocon and the nascent Ii chain inserted into the membrane. The hydrophobic signal anchor sequence is shown as a black box. The position of the photo-activatable (Tmd)Phe is indicated by a star. (**C**) Crosslinking of Ii translocation intermediates that carry the crosslinker at position 119, 121, 123 or 126. Translocation intermediates of IiF*^{119} (lanes 1 and 2), IiF*^{121} (lanes 3 and 4), IiF*^{123} (lanes 5–8) and IiF*^{126} (lanes 9 and 10) of 187 amino acids in length were synthesized *in vitro* in the presence of suppressor tRNA charged with (Tmd)Phe (F*) and rough microsomes (RM). Where indicated, glycosylation was inhibited by the addition of the acceptor tripeptide (AP) benzoyl-NLT-methylamide (lanes 7–10). One half of each sample was irradiated with UV light. Microsomes were extracted with sodium carbonate and samples were analysed for crosslinked products by SDS–PAGE and phosphorimaging. IiF*/187 marks the position of the non-glycosylated translocation intermediate with incorporated (Tmd)Phe. Those forms of Ii that result from termination of translation at the position of the amber stop codon are designated as Iistop. IiF*/187g and Iistop g mark the respective glycosylated forms. Stars indicate IiF*/187(g) crosslinked to a 7 kDa component.

Does nascent chain elongation affect crosslinking to RAMP4?

To test whether nascent Ii chains of different length remain in contact with RAMP4, we synthesized translocation intermediates of various lengths (155–216 amino acids) and identified crosslinked components. Glycosylation of the intermediates was prevented by adding a glycosylation acceptor tripeptide or by using an Ii mutant (IiQ114/120; see Figure 6) that cannot be glycosylated. Crosslinking to the Ii proteins of various length is shown in Figure 5. The minimal Ii length required for crosslinking to RAMP4 from position F*^{123} was 170 amino acid residues (Figure 5, lane 9). All longer translocation intermediates of IiF*^{123} could also be crosslinked to RAMP4 (Figure 5, lanes 10–27) although the longest intermediate of 208 amino acids showed a significantly reduced crosslinking efficiency, 14% as compared with the high crosslinking efficiency of 30–40% for intermediates of 170–203 amino acids in length (see bottom of Figure 5). No crosslinking to RAMP4 was obtained for completed Ii of 216 amino acids (lane 29). Anti RAMP4 antibodies were used to confirm the identity of the component crosslinked to Ii nascent chains of 170, 187 and 203 in length (data not shown). We conclude that the nascent chain at position IiF*^{123} remains in proximity to RAMP4 during different stages of translocation. Only after completion of translocation is the interaction with RAMP4 broken.

We also determined the minimal length of Ii that is once or twice glycosylated. For this we synthesized Ii intermediates as above but allowed their glycosylation. We found that nascent chains up to 175 amino acid residues were not glycosylated, those with 180 residues were once glycosylated and those with 190 and more amino acid residues twice glycosylated (Figure 5, table at the bottom). Thus glycosylation occurs ~10 residues after the interaction of Ii with RAMP4 and 66 residues downstream of the peptidyl transferase centre (Figure 5, table).

Requirements for RAMP4 interaction with Ii

To investigate whether the two N-glycosylation sites of Ii are important for the interaction of Ii with RAMP4, we modified the glycosylation sites. The asparagine (N) residue of the N-glycosylation consensus site is the acceptor amino acid for the oligosaccharide moiety (Kornfeld and Kornfeld, 1985). The X in the N-X-T/S motif can be any amino acid except proline (Bause, 1983). Mutants were generated that contained a glutamine (Q) instead of the asparagine at the second or both glycosylation sites (Figure 6A). In another set of mutants, the glycosylation sites, one or both, were rendered non-functional by inserting a proline (P) at the X position or by exchanging the complete amino acid sequence of the glycosylation consensus site (Figure 6A). Translocation intermediates of the various mutants were tested for

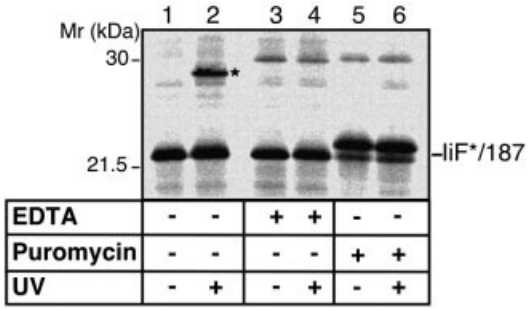

Fig. 2. Release of IiF*^{123}/187 from the ribosome and crosslinking. IiF*^{123}/187 was synthesized and crosslinking induced before (lane 2) or after release of the nascent chain from the ribosome by treatment with EDTA (lane 4) or puromycin (lane 6). Proteins were characterized by SDS–PAGE and phosphorimaging. The star indicates IiF*/187 crosslinked to the 7 kDa component.

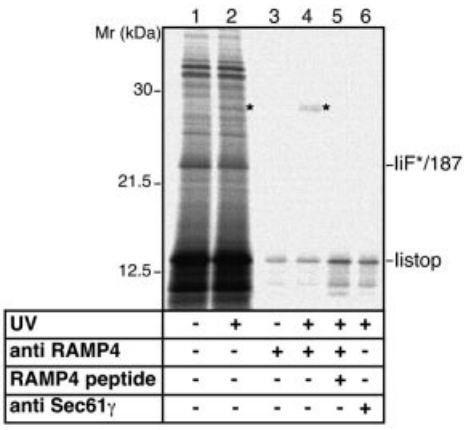

Fig. 4. Immunoprecipitation of the 7 kDa component crosslinked to IiF*^{123}/187. Translocation intermediates of IiF*^{123}/187 were prepared in the presence of the acceptor tripeptide benzoyl-NLT-methylamide. Samples as indicated were irradiated with UV light (lanes 2 and 4–6). After extraction with high salt membranes were either prepared for SDS–PAGE (lanes 1 and 2) or subjected to immunoprecipitation with anti-RAMP4 (1–34) antiserum (lanes 3–5) or an antiserum directed against Sec61γ (lane 6). Ten microgrammes of a competing peptide (RAMP4 1–34) was present in the sample shown in lane 5.

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RAT           MVAKQRIR-MANEKHSKNITQRGNVAKTSRNAPEEKASVGPWL-LALFIFVVCGSAIFQIIQSIRMG--M
MOUSE         MVAKQRIR-MANEKHSKNITQRGNVAKTSRNAPEEKASVGPWL-LALFIFVVCGSAIFQIIQSIRMG--M
HUMAN         MVAKQRIR-MANEKHSKNITQRGNVAKTSRNAPEEKASVGPWL-LALFIFVVCGSAIFQIIQSIRMG--M
C.BRIGGSAE    MAPKQRMA-LANKQFSKNVNNRGNVAKSLK-PAEEKYPAAPWL-IGLFVFVVCGSAVFEIVRYVKMG--W
C.ELEGANS     MAPKQRMT-LANKQFSKNVNNRGNVAKSLK-PAEDKYPAAPWL-IGLFVFVVCGSAVFEIIRYVKMG--W
RICE A.       MTTSRRLADRKSAKFQKNITKRGSVPETTVKKGND-YPVGPMV-LGFFIFVVIGSSLFQIIRTATSGGMA
RICE B.       MTTSRRLSDRKVARFEKNVTKRGSVPET-VKKGND-YPVGPIV-LGFFVFVVVGSSLFQIIRTAQNAGYF
A.THALIANA    MTTSKRLADRKIEKFDKNILKRGFVPETTTKKGKD-YPVGPIL-LGFFDXVVIGSSSFQIIRTATSGGMA
S.CER(YSY6)   MAVQTPRQRLANAKFNKNNEKYRKYGKKKEGKTEKTAPVISKTWLGILLFLLVGGGVLQLISYI-----L
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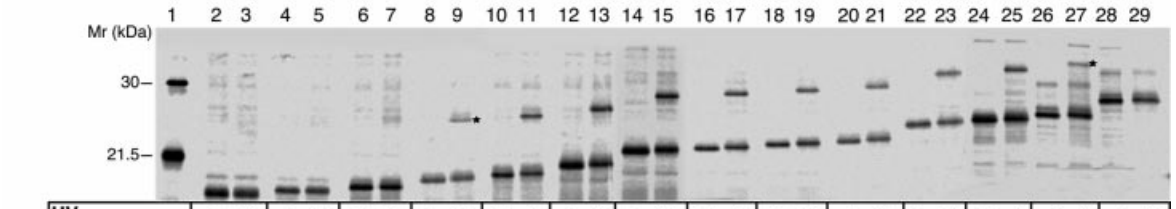
Fig. 3. Amino acid sequences of RAMP4 from different species. RAMP4 was isolated from dog pancreas and the N-terminal sequence determined (underlined). Oligonucleotides were used to select a cDNA from a rat cDNA library. The RAMP4 sequence deduced from a rat cDNA clone is shown and aligned with homologous sequences from several species. Identical amino acids are indicated by a star and similar ones by a dot. A stretch of hydrophobic amino acid residues, the predicted membrane spanning region, is indicated by a solid bar.



UV	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +
Length (aa)	155	160	165	170	175	180	185	187	189	191	198	203	208	216	
Distance from peptidyltransferase	32	37	42	47	52	57	62	64	66	68	75	80	85		
% crosslinked	n.d.	n.d.	n.d.	32	30	32	40	37	30	33	40	27	14	n.d.	
Sites glycosylated	0	0	0	0	0	1	1	1	1	1- 2	2	2	2	2	

Fig. 5. Crosslinking of IiF*^{123} translocation intermediates of different lengths. IiF*^{123} chains of increasing length and full length IiF*^{123} that cannot be glycosylated were synthesized from their respective truncated mRNAs in the presence of microsomal membranes. Crosslinking was induced and samples analysed by SDS–PAGE and phosphorimaging. Stars indicate the shortest and longest IiF* intermediate crosslinked to RAMP4. The length of each translocation intermediate and full length IiF* are given as the number of amino acids. Similarly the calculated distance from the peptidyltransferase centre is given. Using Ii translocation intermediates that can be glycosylated, the glycosylation state of the various nascent Ii chains was derived from their mobility in SDS gels. The number of N-glycosylated sites (1 or 2) or no glycosylation (0) is indicated in the table below the figure.

crosslinking to RAMP4. None of the mutants in the glycosylation consensus sites prevented the interaction with RAMP4 (Figure 6B). The identity of the crosslinked partner, RAMP4, was confirmed by immunoprecipitations for all the mutants (data not shown). Thus crosslinking of Ii to RAMP4 does not depend on functional N-glycosylation sites.

Characterization of a RAMP4-interacting site (RIS) in Ii

As crosslinking of RAMP4 to Ii was rather efficient (30–40%, Figure 5), this suggested a tight, hydrophobic interaction (Martoglio and Dobberstein, 1996). We therefore inspected the region C-terminal of the two glycosylation sites for the presence of hydrophobic amino acid residues. Doublets of hydrophobic amino acid residues were found at positions 126/127 (VM) and 129/130 (LL) and also further downstream at residues 138/139 (VY). The segment comprising residues 121–133 is predicted to form an amphipathic α helix (Figure 7A) (Chou and Fasman, 1978). Two strategies were used to characterize the Ii-RIS. We deleted the segment comprising amino acid residues 124–131 containing the first two doublets of hydrophobic amino acid residues and exchanged each of the hydrophobic amino acid doublets for serines. (Tmd)Phe (F*) was incorporated at position 123 and a translation intermediate of 185 amino acid residues (IiF*/185) inserted into membranes and tested for crosslinking to RAMP4. The Ii mutant lacking amino acids 124–131 (IiF*^{123}Δ124–131) showed no significant crosslinking to RAMP4 in contrast to IiF*^{123}/185 (Figure 7B, lanes 3 and 4). Instead, a set of proteins with estimated molecular weights ~50–60 kDa was crosslinked. Note that this mutant was not glycosylated probably because of its shortening by the deletion of eight amino acid residues. Mutants in which the single doublets VM_{127} (IiF*^{123}VM/SS) or LL_{130} (IiF*^{123}LL/SS) were exchanged for SS showed reduced crosslinking to RAMP4 (~12% crosslinking efficiency) (Figure 7B, lanes 5–8). When both hydrophobic doublets, VM_{127} and LL_{130}, were exchanged for SS (IiF*^{123}VMLL/SSSS), the crosslinking efficiency to RAMP4 was drastically reduced to 6% (Figure 7B, lanes 9 and 10). This indicates that the hydrophobic amino acid residues VM_{127} and LL_{130} are crucial for RAMP4 interaction with Ii. The mutant in which VY_{139} was exchanged for SS (IiF*^{123}VY/SS) showed a slightly reduced level (25%) of crosslinking to RAMP4 (Figure 7B, lanes 5 and 6). This indicates that an extended region C-terminal of the two glycosylation sites contributes to the interaction with RAMP4.

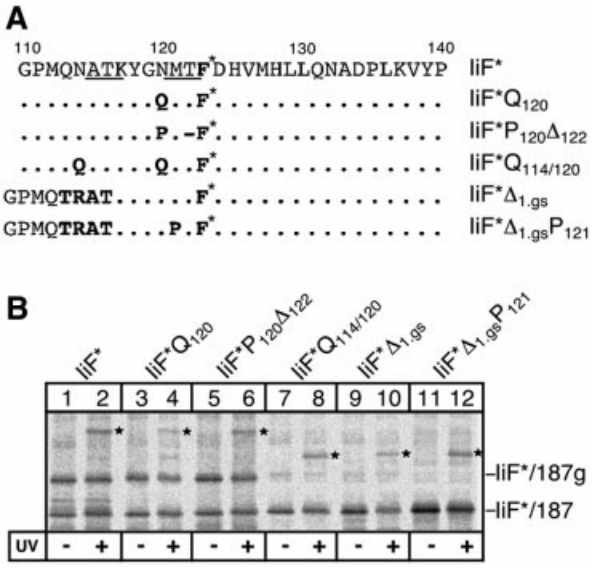


Fig. 6. Crosslinking of mutant IiF*^{123}/187 molecules lacking one or both glycosylation sites. (**A**) Sequences of glycosylation site mutants of IiF*^{123}. Amino acid changes in the mutants are indicated by bold letters. (**B**) Crosslinking of glycosylation site mutants of IiF*^{123}. Translocation intermediates of 187 amino acids were produced and half of each sample irradiated with UV light (+). Samples were analysed by SDS–PAGE and phosphorimaging. Crosslinks to RAMP4 are indicated by a star. Ii of 187 amino acid residues (IiF*/187) and its once glycosylated form (IiF*/187g) are indicated.

N-glycosylation of Ii and mutant Ii molecules

As the Ii-RIS is located C-terminal of the two Ii glycosylation sites and mediates pausing of translocation, it is

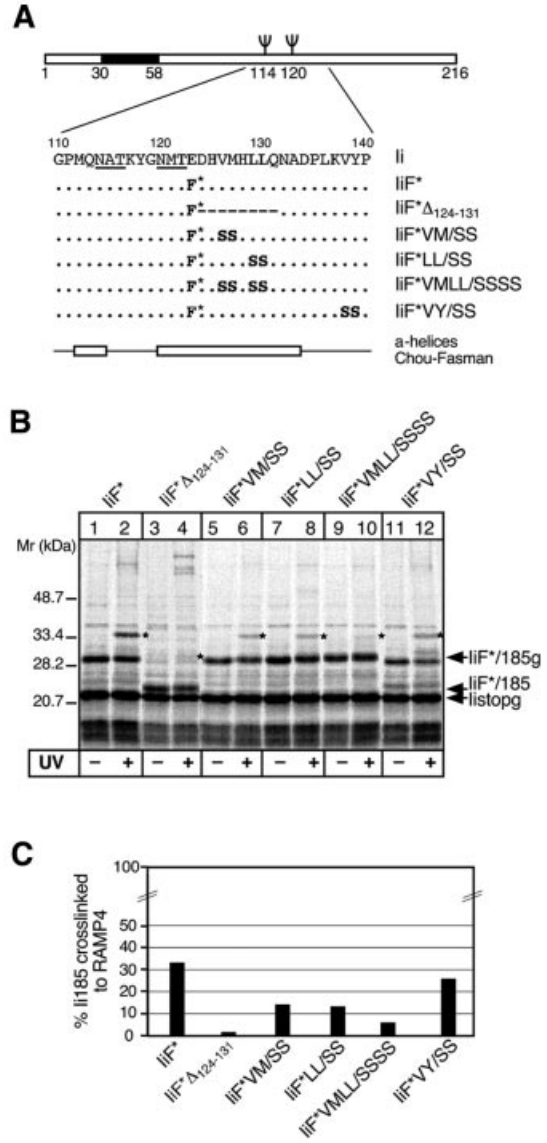


Fig. 7. Characterization of the RAMP4-interacting site in Ii by mutant analysis. (**A**) Linear outline of invariant chain (Ii) and amino acid sequences of the region in which (Tmd)Phe (F*) was incorporated and a deletion or amino acid changes were introduced. The site at which the UV-activatable crosslinker (Tmd)Phe was incorporated is indicated by F*. Doublets of hydrophobic amino acid residues were changed to serines (SS). The two N-glycosylation sites NXT are underlined. Predicted α-helical regions are indicated by open boxes. (**B**) *In vitro* synthesis and membrane insertion of Ii and mutant Ii proteins containing (Tmd)Phe (F*) at position 123. After translation and membrane insertion one half of the sample was UV irradiated (+) to activate the crosslinker. Proteins were then separated by SDS–PAGE and labelled proteins visualized by phosphorimaging. Iistopg, glycosylated Ii proteins released at the stop codon at position 123; IiF*/185, IiF* proteins of 185 amino acids; IiF*/185g, glycosylated form of IiF*/185. Crosslinking adducts between IiF* and RAMP4 are indicated by a star. (**C**) Quantification of the amount of Ii and mutant Ii molecules crosslinked to RAMP4.

conceivable that the interaction with RAMP4 affects glycosylation efficiency. To test this, we expressed Ii and the mutant Ii proteins described above (without the amber codon changes) in HeLa cells. HeLa cells were transiently transfected with expression plasmids encoding either Ii or IiΔ124–131. Cells were pulse labelled for 5 min and then chased with or without cycloheximide in complete medium

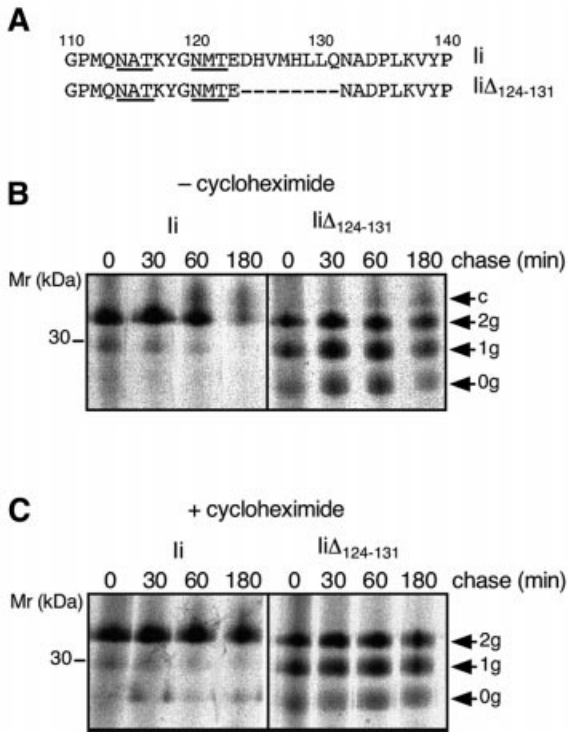


Fig. 8. Glycosylation of Ii and IiΔ124–131 in HeLa cells. (**A**) Ii and IiΔ124–131 sequences. Pulse labelling of Ii and IiΔ123–13 and chase in (**B**) the absence (–) or (**C**) presence (+) of cycloheximide for the times indicated in the figure. Ii and IiΔ123–131 were immunoprecipitated, separated by SDS–PAGE and visualized by phosphorimaging. The non- (0g), mono- (1g) and di-glycosylated (2g) forms of Ii or IiΔ123–131 as well as complex-type glycosylated forms (c) are indicated.

for the times indicated in Figure 8. Cycloheximide inhibits protein synthesis and thereby also the synthesis of unlabelled Ii chains during the chase period. The interrupted synthesis of Ii during the chase period leads to a prolonged retention of Ii in the ER. This is probably due to a less efficient trimerization of Ii when no further proteins are synthesized, as shown by delay of complex-type sugar addition (unpublished observation).

Ii, as expected, was synthesized in HeLa cells in the absence or presence of cycloheximide mainly as a di-glycosylated protein of 33 kDa (Figure 8B and C). In the absence of cycloheximide, complex-type carbohydrate side chains are acquired after 60 min of chase, indicating transport through the Golgi complex (Figure 8B). In the presence of cycloheximide, little modification by complex-type oligosaccharides is seen consistent with prolonged retention of Ii molecules in the ER (Figure 8C).

Ii chains lacking amino acids 124–131 (IiΔ124–131) displayed molecular weight forms of 27, 30 and 33 kDa, representing non-glycosylated, mono-glycosylated and di-glycosylated IiΔ124–131 chains, respectively (Figure 8B). The ratio between non-, mono- and di-glycosylated IiΔ124–131 proteins was not changed during the chase period and was also not affected by the addition of cycloheximide to the chase medium (Figure 8B). We conclude from these experiments that residues 124–131 are crucial for efficient glycosylation of Ii. Incompletely glycosylated Ii chains cannot be additionally glycosylated post-translationally even when they are retained in the ER

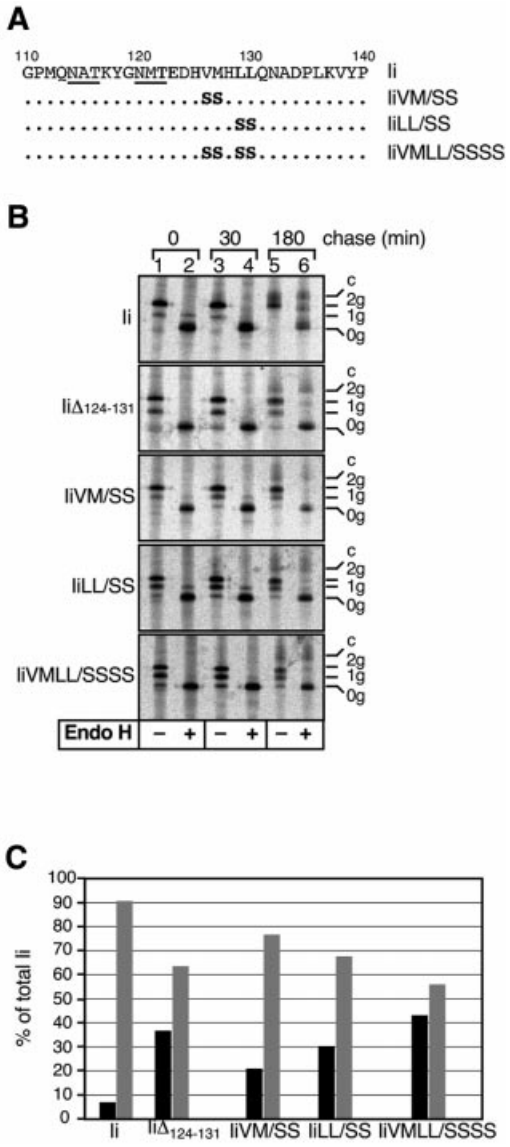


Fig. 9. Pulse–chase labelling and glycosylation of Ii and Ii serine exchange (SS) mutants. (**A**) Sequences of Ii serine exchange (SS) mutants. (**B**) Pulse–chase labelling of Ii, IiΔ123–131, IiVM/SS, IiLL/SS, IiVMLL/SSSS for the times indicated. After immunoprecipitation one half of each sample was treated with Endo H to remove high mannose-type oligosaccharides. Glycosylated forms of Ii are indicated as in Figure 8. (**C**) Quantification of fully glycosylated (grey bars) or non- and mono-glycosylated Ii molecules (black bars) expressed as % of total Ii or mutant Ii molecules synthesized.

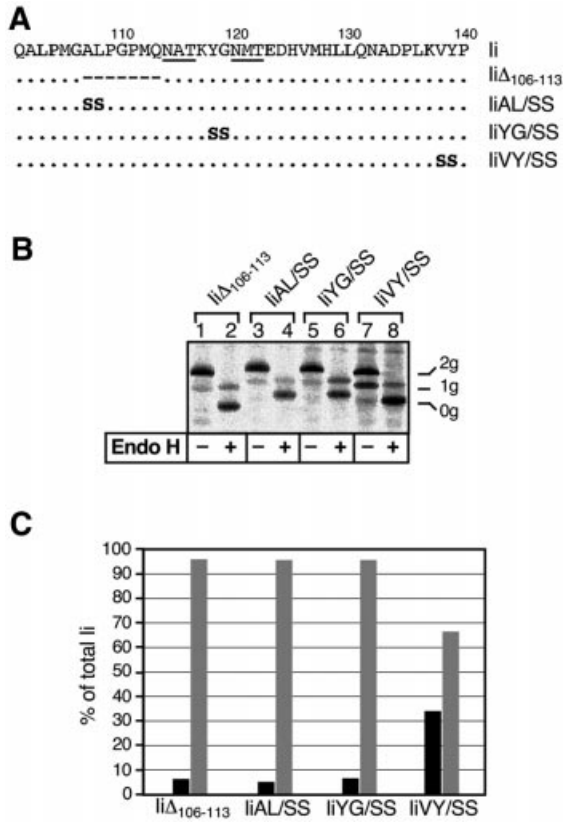


Fig. 10. Pulse labelling and glycosylation of Ii mutants. (**A**) Sequences of Ii mutants. (**B**) Pulse labelling and immunoprecipitation of Ii mutants with (+) or without (–) Endo H treatment. Glycosylated forms are indicated as in Figure 8. (**C**) Quantification of the fully (di)-glycosylated (grey bars) or non- and mono-glycosylated Ii proteins (black bars) shown in (B).

for a prolonged time by a block in protein synthesis (+ cycloheximide).

Expression and glycosylation of Ii and Ii mutants in which several hydrophobic amino acids were exchanged for serines (IiVM/SS; IiLL/SS; IiVMLL/SSSS; Figure 9A) were investigated by pulse–chase labelling, endoglycosidase H (Endo H) treatment and immunoprecipitation. Endo H cleaves high mannose-type carbohydrates but not those containing complex-type carbohydrates. At 0 and 30 min of chase nearly all 33 kDa Ii chains were cleaved to Ii chains with a reduced molecular weight of 27 kDa. This confirms that the 27 kDa form of Ii is non-glycosylated Ii (Figure 9B). After 180 min of chase a substantial amount of Ii chains accumulated with complex-type oligosaccharide side chains as shown by their resistance to Endo H. Ii mutants with one or two of the doublets of hydrophobic amino acid residues exchanged to serines (IiVM/SS, IiLL/SS and IiVMLL/SSSS) showed increased levels of non- and mono-glycosylated Ii forms (Figure 9B). A quantitative analysis revealed the following percentages of underglycosylated Ii chains (Figure 9C): IiΔ124–131 (37%), IiVM/SS (21%), IiLL/SS (30%), IiVMLL/SSSS (42%) (Figure 9C). In contrast, only ~5% of Ii was underglycosylated. This indicates that amino acid residues 124–131 of Ii, and more specifically the above exchanged amino acid residues affect Ii glycosylation.

To test how mutations in other parts affect N-glycosylation of Ii, we generated mutants in which a segment of eight amino acid residues was deleted (IiΔ106–113) and we exchanged three doublets of hydrophobic amino acid residues (AL_{108}, YG_{119} and VY_{139}) for serines (Figure 10A). Mutant Ii proteins were expressed in HeLa cells. One half of the pulse-labelled proteins was treated with Endo H and Ii proteins were immunoprecipitated and characterized by SDS–PAGE and phosphorimaging (Figure 10B). The deletion of eight amino acid residues (IiΔ106–113) and the exchanges by serines (IiAL/SS, IiYG/SS) N-terminal of or between the two N-glycosylation sites did not lead to an increase in underglycosylated Ii chains (Figure 10B). All three mutants yielded <10% underglycosylated Ii chains similar to Ii (Figure 10C). However, changing VY_{139} (IiVY/SS) downstream of the two glycosylation sites to serines led

to an increase in underglycosylated Ii chains. As shown above (Figure 7A and B), this mutant also displayed reduced crosslinking to RAMP4.

Discussion

Translocation of proteins across the ER membrane occurs through an aqueous channel in the translocon. This was deduced from quenching of fluorescent probes site-specifically placed into translocating proteins (Johnson, 1993). Site-specific crosslinking furthermore revealed that the actual protein-conducting channel is lined by proteins of the Sec61p complex (Mothes *et al.*, 1994; Martoglio *et al.*, 1995; Laird and High, 1997). From the low efficiency of crosslinking to Sec61α it has been deduced that hydrophilic parts of translocating nascent chains only occasionally contact the walls of the channel (Martoglio and Dobberstein, 1996). We show here that this is not the case for all translocating parts of proteins. A region in the MHC class II-associated Ii chain that is located downstream of two N-glycosylation consensus sites interacts with RAMP4 during passage through the translocon. The finding that as much as 30–40% of the Ii translocation intermediates can be crosslinked to RAMP4 suggests that the interaction between Ii and RAMP4 is of high affinity and probably of hydrophobic nature. This can be deduced from the chemical property of the crosslinking reagent. Upon light activation the diazarine moiety of the crosslinker is converted to a very short-lived highly reactive carbene that is quenched by the presence of water molecules (Brunner, 1989). Thus a high crosslinking efficiency indicates a tight interaction from which water is largely excluded (Martoglio and Dobberstein, 1996).

The Ii-RIS is shown to be located in a region C-terminal of the two Ii glycosylation sites. The interaction with RAMP4 persisted when nascent chain length was increased from 170 to 208 amino acid residues consistent with continued interaction with RAMP4 during nascent chain elongation. Glycosylation sites themselves were not required for the interaction of Ii with RAMP4. We have identified essential amino acid residues in the Ii-RIS. Deletion of residues 124–131 or changes of two doublets of closely spaced hydrophobic amino acid residues between amino acids 126 and 130 to serine residues resulted in drastically reduced crosslinking to RAMP4. This identifies the Ii-RIS and suggests a hydrophobic interaction between the Ii-RIS and RAMP4. Whether hydrophobicity is the sole determinant for RAMP4 interaction remains to be seen.

Using a systematic crosslinking approach, Rapoport and coworkers (Mothes *et al.*, 1994) have determined the number of amino acid residues between the peptidyl-transferase centre (P-site) in a ribosome and the cytoplasmic and the lumenal side of the translocon, respectively. According to these results, a hydrophilic nascent polypeptide spans the translocon between amino acids 30 and 70 from the P-site. In the case of Ii, we observe crosslinking to RAMP4 at the earliest when the nascent Ii is ~170 amino acids in length (Figure 5). Considering that the crosslinker is placed at residue 123, this gives a distance of 47 amino acid residues between the P-site in the ribosome and the site in Ii that contacts RAMP4. According to these measurements and previous calculations, the translocating polypeptide would interact with RAMP4 in the middle or the end of the translocation channel. As the interaction persists until ~40 more amino acid residues have been polymerized, this indicates a transient retention of the nascent chain in the translocon.

The first site of Ii becomes glycosylated when 180 amino acid residues have been polymerized. At this stage the first glycosylation site is 66 amino acid residues away from the P-site in the ribosome (Figure 5). Nilsson and von Heijne have previously found that the minimal distance of a functional glycosylation site from an internal signal anchor sequence is ~10 amino acid residues (Nilsson *et al.*, 1994). Taken together with the above measurements this places the site of interaction between Ii and RAMP4 close to the lumenal side of the translocon.

RAMP4

Partial protein sequencing and the analysis of cDNAs provided sequence information of a mammalian RAMP4. RAMP4 from rat consists of 66 amino acid residues and has a calculated molecular weight of ~7 kDa. Searching data libraries revealed homologues in other mammals, worms (*Caenorhabditis elegans*), plants (rice) and yeast (*Saccharomyces cerevisiae*). RAMP4 is a membrane protein that exposes its N-terminal hydrophilic portion on the cytoplasmic side and spans the membrane close to the C-terminal end. This was revealed by protease digestion of rough microsomes (K.Schröder and B.Dobberstein, unpublished observation).

RAMP4 has previously been shown to belong to the set of proteins that remains associated with membrane-bound ribosomes upon solubilization with the mild detergent digitonin (Görlich and Rapoport, 1993). RAMP4 and translocon components can be released from ribosomes by treatment with puromycin and high salt (Görlich and Rapoport, 1993). Under these conditions RAMP4 was found in several fractions. The majority was clearly separated from the Sec61p complex, but a small amount consistently fractionated with the Sec61p complex. From this finding it has already previously been speculated that RAMP4 may have a catalytic or regulatory function in the translocation process (Görlich and Rapoport, 1993). The yeast homologue of RAMP4, YSY6p, has been found in a screen for yeast genes that suppress a defective *secY* gene in *E.coli* (Sakaguchi *et al.*, 1991). SecY is the bacterial homologue of Sec61α. YSY6p was shown to improve the translocation of OmpA, the *E.coli* outer membrane protein. YSY6 is found associated with the translocon in yeast ER membranes. Deletion of YSY6p, however, does not result in an obvious phenotype (E.Hartmann, unpublished observation). Further work is required to show the functions of the RAMP4-like proteins in other species.

Other proteins interacting with translocating polypeptides

Transient interactions between nascent secretory proteins and translocon components have been described previously by the group of Lingappa (Hegde and Lingappa, 1997). The interacting sequences were termed pause transfer sequences as they led to a pausing of membrane translocation of certain secretory proteins (Chuck and Lingappa, 1992). Apolipoprotein B (apo B), for example, was found to stop and restart its translocation at several discrete

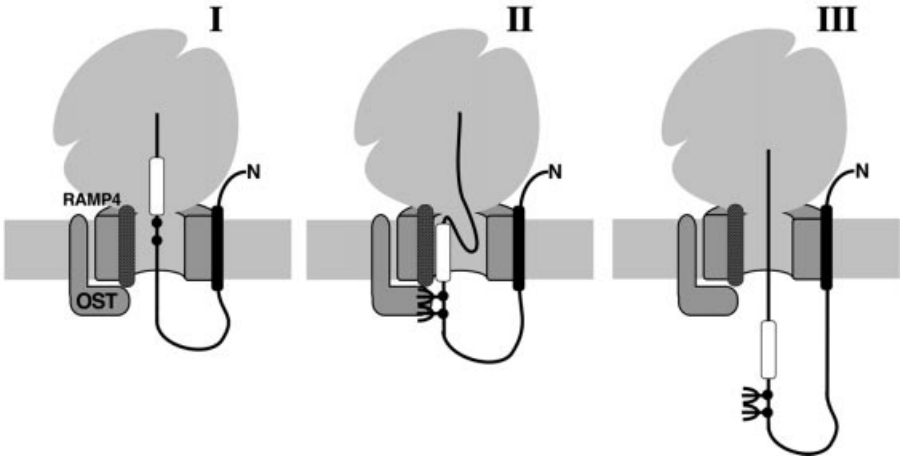


Fig. 11. Hypothetical model of control of Ii chain glycosylation by RAMP4. Three stages of Ii insertion into the membrane are depicted. (**I**) Nascent Ii inserts into the translocon in a loop-like fashion. (**II**) When ~170 amino acid residues have been polymerized Ii interacts via its RIS (white box) with RAMP4. (**III**) Translocation is arrested and OST brought into position to efficiently glycosylate Ii. Upon chain completion, Ii detaches from RAMP4. Oligosaccharides are shown as forked structures.

points of chain elongation due to the function of pause transfer sequences (Chuck *et al.*, 1990). One of these pause transfer sequences has been characterized in more detail. It comprises 10 amino acid residues that were shown to be sufficient to confer translocational pausing on to an otherwise continuously translocated secretory protein (Chuck and Lingappa, 1993). A characteristic feature of this sequence is the high abundance of charged residues. A substitution for either a positively charged amino acid or a threonine abolished pausing. Pause transfer sequences were suggested to contain a structural motif in which positively charged and hydroxylated amino acids are key residues (Chuck and Lingappa, 1993). Comparing the essential features of a pause transfer sequence with those of the Ii-RIS it is clear that the Ii-RIS identified here is distinctly different from an apo B pause transfer sequence. This is further underlined by the finding that the protein, which can be crosslinked to a pause transfer sequence, is different from the RAMP4 protein. Antibodies against RAMP4 failed to recognize the component crosslinked to nascent apo B (Hegde and Lingappa, 1996). Furthermore, the protein crosslinked to a pause transfer sequence had an apparent molecular weight of 11 kDa (Hegde and Lingappa, 1996). In comparison, RAMP4 has a molecular weight of 7 kDa. It thus appears that there exist several types of recognition sequence that can be contacted by different small translocon proteins. The contact between a nascent chain and these translocon components could result in structural changes of the translocon, various times of retention of nascent chains in the translocon or the recruitment of accessory factors that may be involved in the folding or modification of the nascent polypeptide (Hegde and Lingappa, 1996). In the case of apo B, it has been speculated that the stepwise lengthening of the nascent chain may serve the progressive assembly of the protein with lipid from the ER membrane (Chuck *et al.*, 1990; Chuck and Lingappa, 1992, 1993).

Ii interaction with RAMP4 and Ii glycosylation

Ii mutants that displayed reduced crosslinking to RAMP4 also showed reduced glycosylation efficiency when expressed in HeLa cells. Up to 30% of such mutant Ii molecules showed no or only a single oligosaccharide attached. Mutant Ii proteins that could efficiently be crosslinked to RAMP4 did not show a reduced glycosylation efficiency. Thus the reduction in glycosylation efficiency is not the result of an overall change in Ii structure by the mutations. How could an interaction of Ii with RAMP4 increase the glycosylation efficiency of Ii? One possibility is that the interaction of the Ii-RIS with RAMP4 increases the residence time of nascent Ii in the translocon where it is accessible for OST for a prolonged time. We show here that the interaction of RAMP4 with the Ii persists when Ii is progressively increased in length. Thus Ii may be retained for an extended time period at a particular site of the translocation pore (Figure 11). This site may be reached by OST. The degree of glycosylation may therefore be regulated by the time the glycosylation sites are exposed to the OST. In this scenario, the Ii-RIS could be considered as a break point in translocation to allow efficient recruitment of OST. Efficient glycosylation would then depend on the amount of RAMP4 available at the translocon and the availability of OST. Ii seems to require cotranslational glycosylation as incompletely glycosylated Ii chains were no longer glycosylated during the 3 h chase period. Even a prolonged residence time in the ER did not lead to an increase in the degree of Ii glycosylation.

Our findings reported here support the view that the translocon is dynamic and that during translocation there is a variable set of proteins that may gain access to particular sequences of nascent polypeptides during their passage across the translocon (Hegde and Lingappa, 1996).

Functional significance of RAMP4-mediated Ii glycosylation

Why could it be important that the glycosylation of Ii is efficient or regulated by the presence of RAMP4 in the translocon? To consider this question we have to look at the function of Ii. Ii chains assemble in the ER with MHC class II molecules and prevent premature peptide binding (Cresswell, 1996). During transport to endosomal compartments, Ii is proteolytically processed and the resulting

fragments released with the exception of the class II-associated invariant chain peptide (CLIP). CLIP occupies the peptide binding groove of the MHC class II molecules until its exchange for antigenic peptides derived from endocytosed material.

What could be the functional importance of differential Ii glycosylation? Variable oligosaccharide side chains on Ii might play a role in the regulation of Ii processing in endosomes and the generation of CLIP. The oligosaccharide side chains in Ii are located in proximity to the CLIP region. Absence or presence of the oligosaccharides could result in altered processing and thus a different CLIP. This speculation is substantiated by the recent identification of an asparaginyl endopeptidase in endosomes of a human B cell line that was shown to play a central role in processing of a microbial antigen (Manoury *et al.*, 1998). Processing of the antigen was prevented by N-glycosylation of the asparagine at the cleavage site (Manoury *et al.*, 1998). Although it is not known whether the asparaginyl endopeptidase is involved in proteolytic processing of Ii, it is conceivable that incompletely glycosylated Ii may be a substrate for the asparaginyl endopeptidase and processed differently than the fully glycosylated Ii.

The degree of protein glycosylation in cells and tissues is more strictly controlled than previously anticipated (Lis and Sharon, 1993). Even certain pathological states are characterized by changes in the carbohydrate structures of cellular glycoproteins. A marked change in the degree of glycosylation of human chorionic gonadotropin has, for instance, been observed in patients with choriocarcinoma (Lis and Sharon, 1993). In this case, the degree of glycosylation is probably determined by the activity of a glycosyl transferase. Another example where differential glycosylation has been observed is the prion protein. Prion strains have been described that are characterized by a specific N-glycosylation pattern in which the two N-glycosylation sites are either both, only one or none used (Collinge *et al.*, 1996). Conceivably, this reflects cell-type-specific differences in glycosylation efficiencies. The physiological relevance remains thus far unclear.

Our finding reported here that N-glycosylation can be individually regulated by an interaction with RAMP4 provides a possible mechanistic explanation for some of the observed microheterogeneity of glycoproteins (Lis and Sharon, 1993). Differential glycosylation may yield proteins with slightly different functions and this may allow cells to respond quickly to changes in physiological conditions. RAMP4-mediated pausing of nascent chain translocation may not only affect glycosylation of proteins. It is conceivable that other modifications or protein folding or oligomerization can be affected by interaction of a nascent polypeptide with RAMP4.

Materials and methods

Plasmids and transcription

Plasmids pGEM4Ii (High *et al.*, 1993a) and pGEM4ZIi coding for Ii under the control of the SP6 promotor were used for site-directed mutagenesis. Codons 119, 121, 123 and 126 of the coding region of Ii were replaced by the TAG codon by overlap extension PCR (Ho *et al.*, 1989) to give pGEM4IiTAG119, pGEM4IiTAG121, pGEM4IiTAG123 and pGEM4IiTAG126. Glycosylation site mutations were introduced into pGEM4IiTAG123 by overlap extension PCR to give pGEM4IiQ120TAG123, pGEM4IiP120Δ122TAG123, pGEM4IiQ114/116TAG123, pGEM4IiΔ1.gsTAG123 and pGEM4IiΔ1.gs/P121TAG123. A *Bam*HI restriction site was inserted 3′ to codon 187 in all the plasmids. After cleavage by *Eco*RI and *Bam*HI, resulting DNA fragments were cloned into *Eco*RI–*Bam*HI linearized pGEM4 to give pGEM4Ii187 plasmids (pGEM4Ii187TAG119, pGEM4Ii187TAG121, etc.).

Plasmids encoding deletion mutants IiΔ124–131 (pGEM4ZIiΔ124–131) and IiΔ106–113 (pGEM4ZIiΔ106–113) were prepared from pGEM4ZIi by using the ExSite™ site-directed mutagenesis kit (Stratagene). Serine exchange mutants (pGEM4ZIiVM/SS, -IiLL/SS, -IiVMLL/SSSS, -IiAL/SS, -IiYG/SS and -IiVY/SS) were prepared from pGEM4ZIi using the QuikChange™ site-directed mutagenesis kit (Stratagene). Primers and reaction conditions were selected as recommended in the instruction manuals. The QuikChange™ site-directed mutagenesis kit was also used to replace the codon for E-123 by the amber (TAG) codon in the plasmids encoding respective mutant Ii to give pGEM4ZIiΔ124–131TAG123, -IiVM/SSTAG123, -IiLL/SSTAG123, -IiVMLL/SSSSTAG123 and -IiVY/SSTAG123. To express Ii and Ii mutants in living cells, the inserts (*Sma*I–*Bam*HI) from the respective pGEM4Z vectors were subcloned into the eukaryotic expression vector pSV51L (Bakke and Dobberstein, 1990) to give pSV51LIi, -IiΔ124–131, -IiVM/SS, -IiLL/SS, -IiVMLL/SSSS, -IiΔ106–113, -IiAL/SS, -IiYG/SS and -IiVY/SS. All plasmids were amplified in *E.coli* and isolated with the Nucleobond™ plasmid purification kit (Machery Nagel).

mRNAs were synthesized *in vitro* using SP6 RNA polymerase (Gilmore *et al.*, 1991). To prepare mRNAs coding for Ii chains of 155 or 198 amino acids in length, relevant pGEM4Ii plasmids were linearized within the coding region with *Afl*II or *Ppu*MI. For mRNAs coding for Ii chains of 187, 189 and 191 amino acids in length, relevant pGEM4Ii plasmids were linearized within the coding region with *Bam*HI, *Xba*I or *Sal*I. To obtain mRNAs encoding Ii chains of all other lengths, the respective regions of pGEM4Ii or pGEM4ZIi plasmids were amplified by PCR using *Pfu* DNA polymerase (Stratagene). The resulting DNA fragments were transcribed *in vitro* with SP6 RNA polymerase at 42°C in the presence of 500 μM m7G(5′)ppp(5′)G (New England Biolabs) (Nilsson and von Heijne, 1993).

Translation and photo-crosslinking

mRNAs were translated *in vitro* in 25 μl wheat germ extract (Erickson and Blobel, 1983) containing microsomal membranes and SRP prepared from dog pancreas (Martoglio *et al.*, 1998), amber suppressor tRNA chemically amino-acylated with (Tmd)Phe (Graf *et al.*, 1998) and [^{35}S]methionine (>1000 Ci/mmol; Amersham) (Martoglio *et al.*, 1995). Translations were performed at 25°C for 15 min. After translation, samples were put on ice and, where indicated, irradiated with UV light (364 nm) for 2 min (Graf *et al.*, 1998).

Analysis of crosslinked products

After translation and crosslinking, microsomes were extracted with high salt by the addition of KOAc to 500 mM and incubation for 5 min on ice. Membranes were recovered by centrifugation through a 100 μl sucrose cushion [500 mM sucrose, 50 mM HEPES–KOH pH 7.6, 500 mM KOAc, 5 mM $Mg(OAc)_2$] at 48 000 r.p.m. for 3 min at 4°C in a Beckman TLA-100 rotor. Salt-extracted membranes were resuspended in 50 μl 100 mM sodium carbonate pH 11, and incubated for 15 min on ice. Membrane proteins were recovered by centrifugation at 55 000 r.p.m. for 10 min at 4°C in a Beckman TLA-100 rotor.

To release nascent chains from ribosomes, puromycin (4 mM) was added to the translation reaction after crosslinking and samples were incubated at 22°C for 10 min. Membranes were then treated with 500 mM KOAc as described above.

For immunoprecipitation, proteins were solubilized in 200 μl buffer containing 10 mM Tris–HCl pH 7.6, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 0.2 mg/ml phenylmethyl sulfoxide. Samples were supplemented with the relevant antibodies and incubated overnight at 4°C (High *et al.*, 1993b). Antigen–antibody complexes were adsorbed to protein A–Sepharose (Pharmacia) and recovered by centrifugation. Antibodies were raised and affinity purified as described (High *et al.*, 1993b) using peptides corresponding to the 34 N-terminal amino acids of RAMP4 and the N-terminal 30 amino acids of Sec61γ (Hartmann *et al.*, 1994). In the competition assay, a peptide corresponding to the N-terminal 34 amino acids of RAMP4 was used at a concentration of 10 μg/ml.

cDNA cloning

A degenerated oligonucleotide corresponding to the N-terminal peptide sequence of the canine RAMP4 (Görlich and Rapoport, 1993) was used

to screen a rat liver cDNA library (Görlich *et al.*, 1992). Two of the isolated positive clones were sequenced on both strands. Sequence analysis was performed using computer programs of the PCGENE package and the BLAST programs of the NCBI e-mail server.

Pulse–chase experiments

HeLa cells were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing glutamate and supplemented with 10% fetal bovine serum, penicillin and streptomycin (all from Gibco-BRL). Cells were grown to 80% confluency in 150 cm^2 cell culture dishes. After harvesting by trypsinization, cells were washed twice with 10 ml/dish HEBS (20 mM HEPES–KOH pH 7.0, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose) and were resuspended in HEBS at a concentration of 10^7 cells/400 µl. For electroporation, 400 µl cell suspension were combined with 50 µg of vector DNA (2 µg/µl) and incubated for 10 min at room temperature. The cell/DNA mixture was transferred to disposable electroporation cuvettes (BTX P/N 620; 2 mm gap; Bio-Rad) and electroporated at 960 µF and 130 V in a Bio-Rad Gene Pulser. The cells were diluted with 15 ml DMEM with supplements (see above), distributed equally to three 22 cm^2 cell culture dishes and cultured overnight. Twenty-four hours after transfection, cells were rinsed with PBS and cultured for 2.5 h in depletion medium (DMEM minus methionine and cysteine; Gibco-BRL). For subsequent pulse labelling, cells were cultured for 5 min with depletion medium containing 100 µCi/ml ^{35}S cell labelling mix (Amersham). The pulse medium was next replaced by chase medium (DMEM containing 5 mM methionine, 5 mM cysteine and, where indicated, 1 mM cycloheximide) and cells were cultured for the indicated chase times. To stop the chase, the medium was removed, the culture dishes were placed on ice and the cells were washed twice with iced PBS. Cells were lysed with 500 µl/dish lysis buffer [20 mM HEPES–KOH pH 7.3, 100 mM NaCl, 5 mM $MgCl_2$ and 1% (w/v) Triton X-100, 10 µg/ml chymostatin, leupeptin, antipain and pepstatin]. After incubation for 15 min at 4°C, the cell lysates were centrifuged for 5 min at 20 000 *g* to remove cell debris and nuclei.

Immunoprecipitation and Endo H treatment

Cell lysates (~500 µl) were pre-cleared by adding 50 µl protein A–Sepharose (Pharmacia) equilibrated in IP buffer [10 mM Tris–HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.2% (w/v) Triton X-100, 0.2 mg/ml phenylmethylsulfoxide]. After incubation for 60 min at 4°C on a turning wheel, the Sepharose beads were removed by centrifugation. Polyclonal anti-Ii serum (anti-IγC) (Lipp and Dobberstein, 1986) (8 µl) was next added to the pre-cleared cell lysate and samples were incubated overnight at 4°C on a turning wheel. The antigen–antibody complexes were adsorbed to protein A–Sepharose (50 µl) and recovered by centrifugation. The Sepharose beads were washed twice with 1 ml IP buffer, twice with 1 ml IP buffer containing 500 mM NaCl and twice with 1 ml 10 mM Tris–HCl pH 7.5 (High *et al.*, 1993a). For Endo H treatment, washed Sepharose beads were resuspended in 50 µl 50 mM sodium citrate pH 5.5, supplemented with 2 µl (2000 U) Endo H (New England Biolabs). After incubation for 1 h at 37°C, the beads were washed with 1 ml 10 mM Tris–HCl pH 7.5. The washed protein A–Sepharose beads were prepared for SDS–PAGE.

Electrophoresis

High salt and sodium carbonate extracted membranes and immunoprecipitated proteins were solubilized in 25 µl sample buffer containing 125 mM Tris–HCl pH 6.8, 5 mM EDTA, 50 mM dithiothreitol, 5% glycerol and 2% SDS. After incubation for 10 min at 65°C, samples were analysed by SDS–PAGE using 12.5, 14 or 12–18% gradient gels (Laemmli, 1970). [^{35}S]methionine-labelled proteins were visualized using a Fuji phosphorimager BAS1000.

Acknowledgements

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