# Signal Sequence–dependent Function of the TRAM Protein during Early Phases of Protein Transport across the Endoplasmic Reticulum Membrane

Sabine Voigt,* Berit Jungnickel,* Enno Hartmann,‡ and Tom A. Rapoport*

*Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115; and ‡Max-Delbrueck-Center for Molecular Medicine, 13125 Berlin-Buch, Germany

***Abstract.*** Cotranslational translocation of proteins across the mammalian ER membrane involves, in addition to the signal recognition particle receptor and the Sec61p complex, the translocating chain–associating membrane (TRAM) protein, the function of which is still poorly understood. Using reconstituted proteoliposomes, we show here that the translocation of most, but not all, secretory proteins requires the function of TRAM. Experiments with hybrid proteins demonstrate that the structure of the signal sequence determines whether or not TRAM is needed. Features that distin-

guish TRAM-dependent and -independent signal sequences include the length of their charged, NH₂-terminal region and the structure of their hydrophobic core. In cases where TRAM is required for translocation, it is not needed for the initial interaction of the ribosome/ nascent chain complex with the ER membrane but for a subsequent step inside the membrane in which the nascent chain is inserted into the translocation site in a protease-resistant manner. Thus, TRAM functions in a signal sequence–dependent manner at a critical, early phase of the translocation process.

T HE transport of proteins across the mammalian ER membrane generally occurs in a cotranslational manner. The process starts in the cytosol with a targeting phase. As soon as the signal sequence of a growing nascent polypeptide chain has emerged from the ribosome, it is recognized by the 54-kD subunit of the signal recognition particle (SRP)[1] (for review see Rapoport, 1992; Walter and Johnson, 1994). The entire complex of ribosome, nascent chain, and SRP then binds to the ER membrane by two separate interactions, one between SRP and its membrane receptor (SRP receptor or docking protein) (Gilmore et al., 1982; Meyer et al., 1982), and the other between the ribosome and a membrane protein complex, the Sec61p complex (Görlich et al., 1992b; Kalies et al., 1994). The latter is likely to be the core component of the translocation apparatus in the ER membrane and is thought to form a protein-conducting channel. Recent data suggest that the nascent polypeptide–associated complex (NAC), a protein complex that is bound to translating ribosomes, also plays a role during the targeting process (Wiedmann

et al., 1994). It may prevent membrane binding of ribosome–nascent chain complexes that do not have bound SRP (Lauring et al., 1995a,b). In the absence of NAC, ribosome–nascent chain complexes can be bound to the membrane independently of SRP or the presence of a functional signal sequence (Jungnickel and Rapoport, 1995; Lauring et al., 1995b).

After the targeting phase, the ribosome is bound initially to the Sec61p complex only weakly; the nascent chain is accessible to protease digestion and can be extracted by high salt concentrations (Jungnickel and Rapoport, 1995). As the nascent chain is elongated during translation, a tighter interaction between the ribosome and the Sec61p complex is attained; now the nascent chain is no longer sensitive to protease or extractable by high salt concentrations. The transition from the weak to the tight membrane interaction of the ribosome–nascent chain complex requires a functional signal sequence (Jungnickel and Rapoport, 1995). This step also appears to lead to the opening of the protein-conducting channel towards the lumen of the ER (Crowley et al., 1994), suggesting that the channel is gated by the signal sequence. Upon insertion of the nascent chain into the translocation site, the elongating nascent chain is likely to be transferred directly from the channel in the ribosome into the protein-conducting channel in the membrane (Simon and Blobel, 1991; Görlich et al., 1992b; Crowley et al., 1993).

Cotranslational protein transport can be reproduced with reconstituted proteoliposomes containing only three

1. *Abbreviations used in this paper:* NAC, nascent polypeptide–associated complex; pPL, preprolactin; ppαF, prepro-α-factor; PK-RM, canine microsomes stripped of ribosomes by puromycin and high salt; SRP, signal recognition particle; TRAM protein, translocating chain–associating membrane protein; VSV G, vesicular stomatitis virus glycoprotein.

purified ER membrane proteins: the SRP receptor, the Sec61p complex, and the translocating chain–associating membrane (TRAM) protein (Görlich and Rapoport, 1993). Whereas the roles of the SRP receptor in the targeting step and of the Sec61p complex in the actual translocation process are beginning to be understood, the function of the TRAM protein is still rather mysterious. TRAM is a glycosylated, multispanning membrane protein that can be purified as a single polypeptide chain (Görlich et al., 1992a). In the reconstituted system, it is stimulatory but not essential for transport of the secretory protein preprolactin, but it is essential for the translocation of prepro-α-factor (ppαF) (Görlich et al., 1992a; Görlich and Rapoport, 1993). Cross-linking experiments carried out for preprolactin (pPL) suggest that TRAM contacts nascent chains during an early phase of the translocation process (Görlich et al., 1992a). TRAM cross-links are seen with nascent polypeptides that are long enough to produce a tight interaction between the ribosome and the Sec61p complex; shorter polypeptides that are associated with weakly bound ribosomes, or nascent chains that carry a nonfunctional signal sequence do not give cross-links to TRAM (Jungnickel and Rapoport, 1995). TRAM contacts mostly the charged, $NH_2$-terminal region of the signal sequence of the nascent chain after its insertion into the translocation site (High et al., 1993; Mothes et al., 1994). The hydrophobic portion of the sequence can be cross-linked to the α subunit of the Sec61p complex and to lipids (Martoglio et al., 1995). Once the signal sequence is cleaved off by the signal peptidase, TRAM can no longer be cross-linked to the nascent chain (Mothes et al., 1994).

These results raise a number of questions: Is TRAM dependence a general phenomenon for secretory proteins? What sequence features determine whether the translocation of a protein is TRAM dependent? At which step in the translocation process does TRAM function? In the present study, we have addressed these questions with the reconstituted translocation system. We demonstrate that the majority of secretory proteins do require TRAM. Using hybrid proteins, we show that the signal sequence determines the requirement for TRAM. Comparison of signal sequences of TRAM-dependent and -independent proteins and mutagenesis of the signal sequence of preprolactin indicate that the charged, $NH_2$-terminal region and the hydrophobic core contain important determinants for TRAM dependence. We demonstrate that TRAM is required during the phase inside the membrane in which the nascent chain is firmly inserted into the translocation site. Based on these results, we conclude that TRAM functions in a signal sequence–dependent manner at a critical, early phase of translocation.

## Materials and Methods

### Plasmids

The following plasmids were used: pGEMBP1, coding for preprolactin (from R. Gilmore, University of Massachusetts, Worcester); pSP64rpGH, coding for rat growth hormone precursor (provided by S. Monier, Max-Delbrueck-Center for Molecular Medicine, Berlin, Germany); pMR48, coding for preKar2p (from M. Rose, Yale University); pSP65αF, coding for ppαF (from E.T. Young, University of Washington); pDM9G, coding for vesicular stomatitis virus (VSV) pre-glycoprotein (from R. Gilmore);

pCA2, coding for a preprocecropin-DHFR fusion protein (from R. Zimmermann, Universität des Saarlandes, Germany); pSP6-LG211, coding for pre-Ig κ chain (from B. Dobberstein, University of Heidelberg, Germany); pSP6-HG201, coding for pre-IgG heavy chain (from B. Dobberstein); pSPIL22, coding for pre-interleukin 2 (from T. Omura, Kyushu University, Japan); pGEM2Tac, coding for pre-interleukin 2-receptor α chain (original clone pCDM8Tac from R. Klausner, National Institutes of Health); pGEM2-suc2-91, coding for pre-invertase (from D. Meyer, University of California, Los Angeles); pGEM2ppαF404, coding for a ppαf mutant with a deletion of the amino acids 32–89 (from J. Rothblatt, Dartmouth College, Hanover, NH and D. Meyer); pCA37, coding for a fusion protein with the signal sequence of preprocecropin linked to the mature prolactin (from R. Zimmermann); pSPSpLgG, coding for a fusion protein of the signal sequence of preprolactin followed by a portion of Escherichia coli β-lactamase and glycoglobin (provided by V. Lingappa, University of California, San Francisco).

### Membranes and Proteoliposomes

Rough microsomes were prepared from dog pancreas and stripped of ribosomes by puromycin/high salt treatment (Walter and Blobel, 1983; Görlich and Rapoport, 1993). The purification of the SRP receptor, the Sec61p complex, and the TRAM protein, as well as their reconstitution into proteoliposomes, have been described previously (Görlich and Rapoport, 1993). For each preparation, the optimal relative concentrations of the individual components in proteoliposomes were determined in titration experiments using preprolactin and ppαF as transport substrates. The efficiency of transport of pPL and ppαF into proteoliposomes containing SRP receptor, Sec61p complex, and TRAM was generally 30–50% and 15–30%, respectively.

### Construction of Plasmids

For construction of pSP65ssαFmPL, a DNA fragment coding for the mature part of preprolactin was amplified by PCR from pGEMBP1 with primers corresponding to the beginning of the mature region of pPL and the downstream SP6 promoter. The PCR product was cut with SalI and cloned into the HincII and SalI sites of pSP65αF.

For construction of pGEM2ssPLmαF, a PstI fragment of pSP65αF coding for part of the signal sequence and the entire mature region of ppαF was cloned into the PstI site of pGEMBP1, behind the sequence coding for pPL. A deletion of the mature part of preprolactin was produced by PCR; a chimeric primer between the COOH-terminal region of the signal sequence of pPL (5') and the $NH_2$-terminal region of pro–α-Factor (3') and a primer to the SP6 promoter were used for amplification. The PCR product was cleaved with PflMI and SalI and the fragment cloned into the PflMI and SalI sites of pGEMBP1.

The sequences of the hybrid proteins as well as the $NH_2$-terminal sequences of all other proteins used in this study were verified by sequencing the corresponding plasmids using SP6 or T7 primers.

### In Vitro Mutagenesis

All signal sequence mutants of pPL described in this paper were produced with the in vitro mutagenesis kit from Promega Corp. (Madison, WI). The plasmid pGEMBP1 was cut with HindIII and EcoRI. The resulting fragment, coding for the untranslated region of β-globin followed by the entire sequence of pPL, was inserted into the phagemid vector pAlter. Mutagenic oligonucleotides were used to generate the deletion mutants pPL Δ2-11, pPL ΔVSN, and pPL ΔSN. Alterations of codons for lysines at positions 4 and 9 into asparagines (pPL N4/9), and/or exchanges of codons for asparagines at positions 36 and 40 into lysines (pPL K36/40 and pPL N4/9 K36/40, respectively) were made in a similar manner. Single-stranded DNA of the plasmid pAlter coding for the mutant pPL N4/9 was used to produce mutants with deletions of 6, 12, 18, 24, or 33 bases, resulting in the mutants pPL Δ2-3, pPL Δ2-5, pPL Δ2-7, pPL Δ2-9, and pPL Δ2-12. All mutants were confirmed by PCR sequencing using SP6 and T7 primers.

### In Vitro Transcription

mRNAs were synthesized by transcription of the various plasmids using T7 or SP6 RNA polymerase. All pAlter plasmids coding for pPL signal sequence mutants were cleaved with EcoRI before transcription with SP6 RNA polymerase. The plasmid coding for invertase was cleaved with BamH1, resulting in a fragment coding for the first 262 amino acids. The mRNA coding for VSVG 90 was synthesized after cutting the plasmid

with AvaII; mRNAs for pPL86 and ssαFmPL86 were produced after cleaving the plasmids with PvuII. The plasmid coding for ppαFΔ32-89 was cleaved with NciI before transcription to generate a truncation within the coding sequence. An NH$_2$-terminal fragment of the kar2 gene coding for 228 amino acids was amplified by PCR using a 3' primer that introduces a stop codon. The PCR fragment was gel purified and transcribed with T7 polymerase.

### Translocation Assays

mRNAs coding for the test proteins were translated in a wheat germ system containing 40 nM SRP, 10 μCi [$^{35}$S-]methionine and 0.1 equivalent (eq.)/μl (for definition of equivalents, see Walter et al., 1981) of puromycin/high salt stripped rough pancreatic microsomes (PK-RM). When proteoliposomes reconstituted from purified membrane proteins were used, the final concentrations in the translation mixture of SRP receptor, Sec61p complex, and TRAM ranged from 0.1–0.3 eq./μl, 0.2–0.4 eq./μl, and 0.1–0.4 eq./μl, respectively. After a 20-min incubation at 26–28°C, the samples were split into two aliquots, one of which was incubated with 0.5 mg/ml proteinase K for 40 min on ice. The proteins were then precipitated with TCA, washed with acetone, and analyzed by SDS-PAGE. The translation mixtures with mRNA coding for truncated invertase were treated with 1 mM puromycin for 5 min on ice followed by 5 min at 26°C to release the nascent chains from the ribosomes before protease digestion. All translocation assays with reconstituted proteoliposomes were carried out at least twice with different batches of purified translocation components.

### Cross-linking and Assays for Membrane Insertion of Nascent Chains

Synthesis of nascent chains, isolation of ribosome–nascent chain complexes, cross-linking, and protease-protection assays were all performed as described (Jungnickel and Rapoport, 1995).
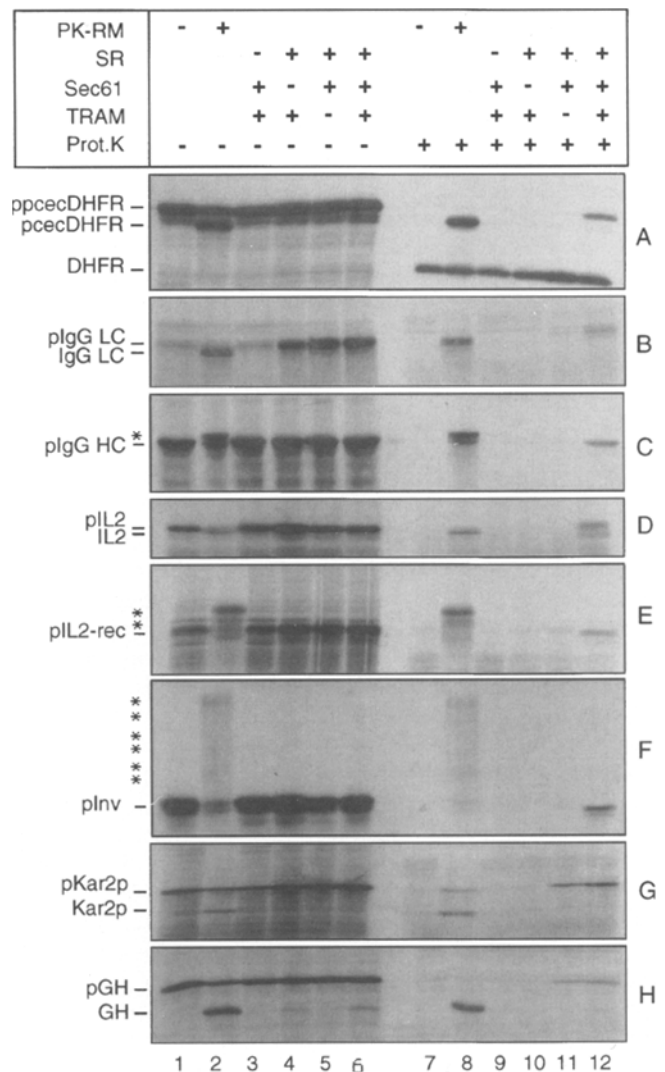
### Gel Electrophoresis

Proteins were normally separated in 13.75% acrylamide gels, except for VSV G protein (10% acrylamide), and immunoglobulin light chain and interleukin 2 (10–20% linear acrylamide gradient gels). For the analysis of membrane insertion of short nascent chains, 10–20% linear acrylamide or 12% Tris/Tricine gels were used. The dried gels were analyzed with a PhosphoImager (Fuji Co., Tokyo, Japan) for quantitation of the radioactivity and subsequently exposed to x-ray film.

## Results

### Translocation of Most Secretory Proteins Requires the TRAM Protein

Previous experiments on a small number of substrates indicated that TRAM is required for the translocation of some, but not all, proteins (Görlich and Rapoport, 1993). To determine whether TRAM dependence is a general phenomenon, we tested a variety of proteins with cleavable signal peptides for their ability to be transported into proteoliposomes containing or lacking TRAM (Fig. 1). The SRP receptor, the Sec61p complex, and TRAM were purified from dog pancreatic microsomes and reconstituted with pure phospholipids resembling the phospholipid mixture of ER membranes (Görlich and Rapoport, 1993). Proteoliposomes were produced that either contained all three components or lacked individual components. The



Figure 1. TRAM is required for the translocation of most secretory proteins. The indicated secretory proteins were synthesized in a wheat germ translation system in the presence of SRP, [$^{35}$S-]methionine and either native microsomes stripped of ribosomes by puromycin/high salt treatment (PK-RM), or reconstituted proteoliposomes. The latter contained different combinations of pu-

rified SRP receptor (SR), Sec61p complex (Sec61), and TRAM protein (TRAM), as indicated. After translation, the samples were split in half; one half was analyzed directly (lanes 1–6) and the other was treated with proteinase K (Prot.K) to digest all non-translocated material (lanes 7–12). The proteins were separated in SDS gels and analyzed by autoradiography. Several experiments were carried out for each protein. The translocation efficiency in the absence of TRAM (given below in parenthesis) was calculated by dividing the percentage of translocation in the absence of TRAM (proteolyzed versus nonproteolyzed sample) by the percentage of translocation in the presence of TRAM, and multiplying this figure by 100. (A) ppcecDHFR, H. cecropia preprocecropinA–DHFR fusion protein (15% ± 1.3); (B) pIgG LC, mouse pre-immunoglobulin κ chain (10% ± 1.1); (C) pIgG HC, mouse pre-immunoglobulinG heavy chain (<5%); (D) pIL2, human pre-interleukin 2 (7% ± 1.5); (E) pIL2-rec, human pre-interleukin 2 receptor alpha chain (<5%); (F) pInv, fragment of 262 amino acids of S. cerevisiae preinvertase (14% ± 2); (G) pKar2p, fragment of 228 amino acids of S. cerevisiae pre-Kar2 protein (36% ± 0.8); (H) pGH, rat pregrowth hormone (74% ± 2.5). Asterisks indicate glycosylated forms of the proteins. The abbreviations pcecDHFR, IgG LC, IL2, Kar2p, GH indicate the signal sequence-cleaved forms of these proteins. The DHFR domain of ppcecDHFR is resistant to proteinase K in the absence of microsomes (A, lane 7). The low level of translation in lanes 1 and 3 of B is due to a more pronounced SRP-induced translational arrest.

vesicles were added to a wheat germ in vitro translation system containing SRP, [$^{35}$S-]methionine, and the mRNA coding for the test protein. After translation, the samples were subjected to a protease protection assay. One-half of the sample served to analyze the total products, the other half was treated with proteinase K to digest all material outside the vesicles; the protected material is considered to be translocated across the phospholipid bilayer. Control reactions were carried out with samples lacking membranes and with native microsomes stripped of ribosomes by treatment with puromycin and high salt (PK-RM).

The results of these experiments show that all proteins tested are transported into proteoliposomes that contain all three translocation components (Fig. 1, lane *12*) but not into vesicles that lack either the SRP receptor (lane *9*) or the Sec61p complex (lane *10*). Taking into account similar results for other proteins tested previously (Görlich and Rapoport, 1993; Oliver et al., 1995), it thus appears that both translocation components are generally essential for the transport of proteins. Most proteins also required the presence of TRAM (lane *11* vs. *12*). This group included proteins that showed different overall translocation efficiencies and originated from various organisms and cells. However, we found two proteins (pre-Kar2p from *Saccharomyces cerevisiae* and pregrowth hormone from rat) whose translocation did not depend on TRAM (*G* and *H*), like that of the previously studied bovine preprolactin. We conclude that the dependence of the translocation reaction on TRAM is not the exception but rather the rule, as most secretory proteins seem to fall into this class. These results also support the previous conclusion (Görlich and Rapoport, 1993) that the three translocation components constitute a minimum translocation apparatus of the ER membrane. The translocation efficiencies (percentage of protease-protected material) differed for the proteins tested (between 18 and 80% for PK-RM and between 10 and 40% for reconstituted proteoliposomes).

## TRAM Dependence Is Determined by the Signal Sequence

To analyze the sequence features that determine TRAM dependence of a secretory protein, we performed a signal sequence swap experiment. We used as examples for TRAM-dependent and -independent proteins ppαF and pPL, respectively (Görlich and Rapoport, 1993). Two hybrid proteins were constructed, one with the signal sequence of ppαF linked to the mature region of pPL, and the other with the signal sequence of pPL linked to the mature region of ppαF (Fig. 2 *A*). Translocation of the wild-type and the hybrid protein constructs was tested as before with proteoliposomes that contained or lacked TRAM (Fig. 2 *B*). The two proteins that contained the signal sequence of preprolactin (panels *A* and *D*) were translocated with about equal efficiency in the absence or presence of TRAM (lanes *11* vs. *12*), whereas proteins that contained the signal sequence of ppαF (panels *B* and *C*) depended on TRAM for translocation. Thus, the signal sequence determines whether the translocation of a protein depends on TRAM.

The behavior of two other hybrid proteins is consistent with this conclusion: the signal sequence of preproce-
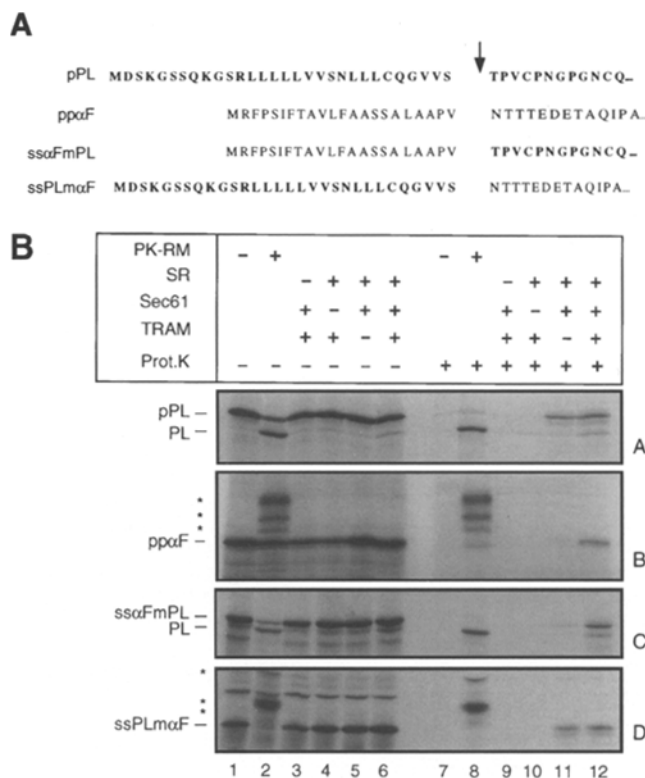


*Figure 2.* TRAM dependence is determined by the signal sequence. (*A*) Sequences of the NH$_2$ termini of bovine preprolactin (*pPL*) and of *S. cerevisiae* prepro-α-factor (*ppαF*), as well as of mutants containing swapped signal sequences. ssPLmαF, hybrid protein containing the signal sequence of pPL linked to the mature region of ppαF; ssαFmPL, hybrid protein containing the signal sequence of ppαF linked to the mature region of pPL. Sequences of pPL are shown in bold face. Arrow indicates the signal sequence cleavage site. (*B*) The wild-type and hybrid proteins were tested for translocation into native microsomes (*PK-RM*) or proteoliposomes as described in Fig. 1. Lanes *1–6* show the total products, lanes *7–12* the translocated material protected against digestion by proteinase K (*Prot.K*). The glycosylated forms of pro–α-factor are marked by asterisks. Note that in the case of ssPLmαF (*panel D*), most of the protein received only two instead of three carbohydrate chains (*compare with panel B*), presumably because one of the glycosylation sites immediately follows the modified signal peptide cleavage site (see *A*). The bands of higher mobility seen in lane 12 are the signal sequence–cleaved forms of preprolactin (prolactin, PL), caused by a contamination of the TRAM preparation with signal peptidase.

cropin A, when linked to the mature region of preprolactin, yielded a TRAM-dependent protein, and the signal sequence of preprolactin, fused to a sequence that consists of portions of α-globin and β-lactamase, was TRAM independent (data not shown).

## Differences in Signal Sequence Structure between TRAM-dependent and -independent Proteins

In what respect do the signal sequences of TRAM-dependent and -independent proteins differ? A comparison of the signal sequences of tested proteins (Fig. 3) raises several possibilities. Perhaps the most obvious difference is the length of the polar, NH$_2$-terminal region (N-domain;

**A**

TRAM - dependence

| Sequence | Protein | |
|---|---|---|
| MRFPSIFTAVLFAASSALAAPV | prepro-α-factor (S.cerevisae) | + |
| MSIQHFRVALIPFFAAFCLPVFA | pre-β-lactamase (E.coli) | + |
| MKCLLYLAFLFIGVNC | pre-glycoprotein (ves.stomatitis virus) | + |
| MNFSRIFFVFACLTALAMVNA | preprocecropin (H.cecropia) | + |
| MDMRVPAHVFGFLLLWFPGTRC | pre-Ig κ-light chain (mouse) | + |
| MDIWIFLFILSGTAG | pre-IgG heavy chain (mouse) | + |
| MYRMQLLSCIALSLALVINS | pre-interleukin 2 (human) | + |
| MDSYLLMWGLLTFIMVPGCQA | pre-IL2-receptor (human) | + |
| MLLQAFLLAGFAAKISA | pre-invertase (S.cerevisae) | + |
| MDSKGSSQKGSRLLLLLVVSNLLLCQGVVS | preprolactin (bovine) | - |
| MAADSQTPWLLTFSLLCLLWPQEAGA | pre-growth hormone (rat) | - |
| MFFNRLSAGKLLVPLSVVLYALFVVILPLQNSFHSSNVLVRG | pre-Kar2p (S.cerevisae) | - |

**B**



*Figure 3.* Sequence comparison between TRAM-dependent and -independent signal sequences (*A*) and hydropathy plots of the NH₂ termini of the proteins (*B*). The latter were calculated with the Kyte-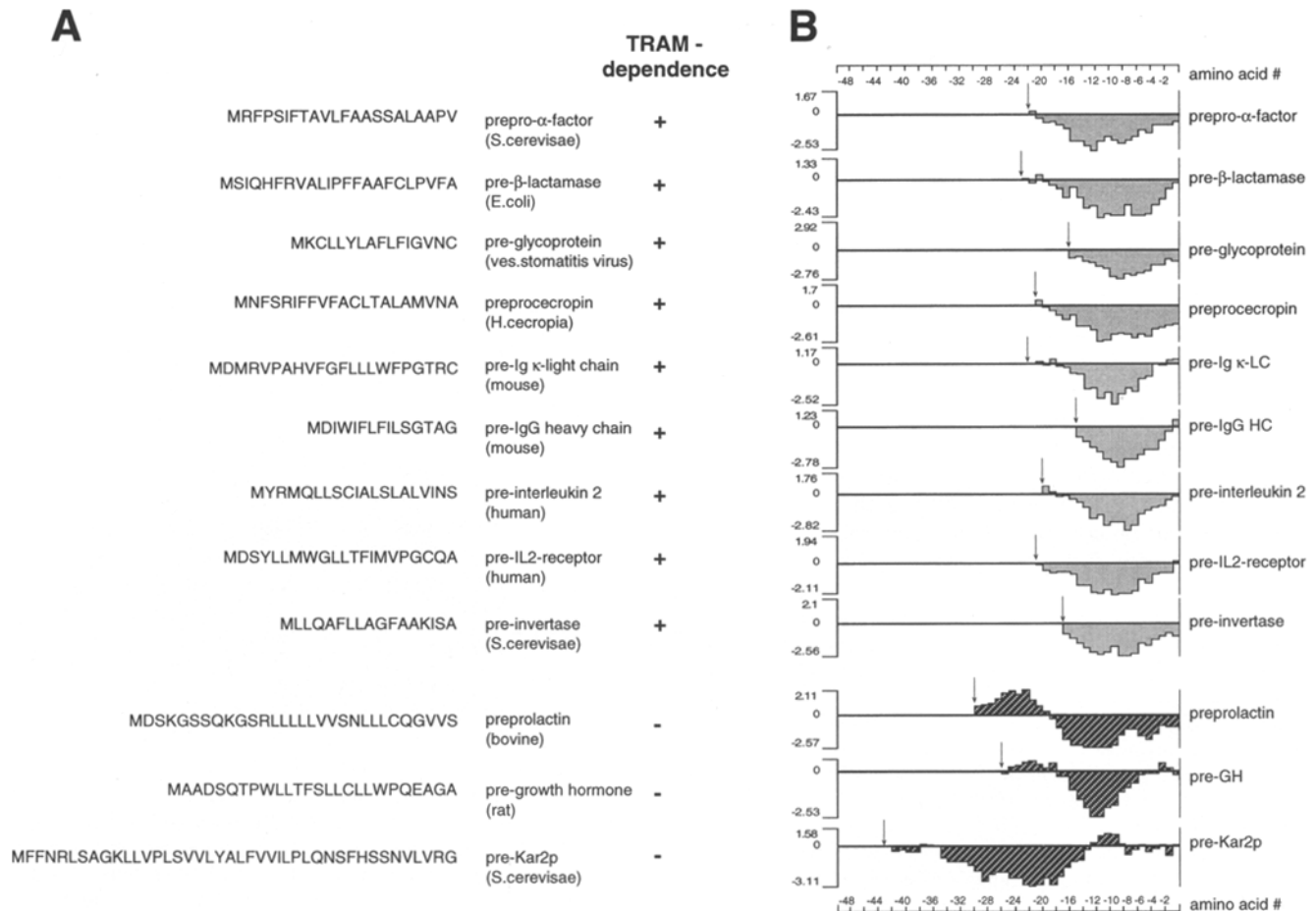Doolittle algorithm using a window of 9 residues. Values above and below zero indicate hydrophilic and hydrophobic regions, respectively. Arrows denote the first amino acid of the signal sequence.

von Heijne, 1985) that precedes the hydrophobic core of the signal sequence. For TRAM-dependent proteins, the hydrophilic N-region is short (up to 5 residues), whereas for TRAM-independent proteins it is significantly longer (at least 9 residues, Fig. 3 *B*). Another possible difference is the amino acid composition of the core region itself. In TRAM-independent proteins, leucine and valine residues predominate in the hydrophobic core, whereas in TRAM-dependent proteins, alanine and phenylalanine residues are more frequent (Fig. 3 *A*). Yet another discriminating feature may be the length of the hydrophobic core region; it tends to be longer for TRAM-independent proteins than for TRAM-dependent ones.

To test the various possibilities suggested by the sequence comparison we used in vitro mutagenesis. Assuming that TRAM-independent proteins have special features that allow them to function even in the absence of TRAM, we attempted to convert the signal sequence of preprolactin into one that depends on TRAM. In these experiments, only those mutants would be instructive that maintained a constant level of translocation in the presence of TRAM.

We first tested the idea that the length of the N-region preceding the hydrophobic core is important. Deletion of almost the entire N-domain (amino acids 2–11 of the signal sequence of preprolactin; pPL Δ2-11; see Fig. 4 *A*) indeed converted the protein into one whose translocation in the absence of TRAM was much reduced but in the presence of TRAM was as high as for the wild type (Fig. 4 *B, panel B*, lane *11* vs. *12*).

Since positive charges in the N-domain may be of particular importance for the function of a signal sequence (Sakaguchi et al., 1992), we were concerned that the increase of TRAM dependence upon deletion of the N-domain could be caused by the loss of two positive charges (see Fig. 4 *A*), rather than by changes of the length. However, when the lysines at positions 4 and 9 were replaced by asparagines (pPL N4/9), the protein did not become more TRAM dependent (Fig. 4 *B, panel C*, lane *11* vs. *12*). Moreover, increased TRAM dependence of pPL Δ2-11 was not the result of an altered balance of the positive charges preceding and following the hydrophobic core of the signal sequence. Even the introduction of two positive charges into the mature part of either wild-type pPL (resulting in the mutant pPL K36/40; Fig. 4 *B, panel D*) or the mutant pPL N4/9 (resulting in pPL N4/9 K36/40; Fig. 4 *B, panel E*), left the protein TRAM independent. It should be noted that rat growth hormone, a TRAM-independent protein, does not
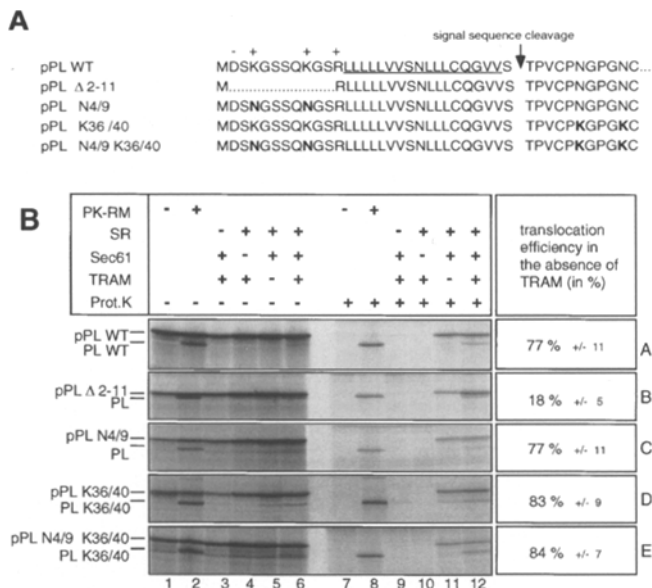
**A**

signal sequence cleavage

| | |
|---|---|
| pPL WT | MDSKGSSQKGSRLLLLLVVSNLLLCQGVVS ↓TPVCPNGPGNC... |
| pPL Δ2-11 | M........................RLLLLLVVSNLLLCQGVVS TPVCPNGPGNC |
| pPL N4/9 | MDSNGSSQNGSRLLLLLVVSNLLLCQGVVS TPVCPNGPGNC |
| pPL K36/40 | MDSKGSSQKGSRLLLLLVVSNLLLCQGVVS TPVCPKGPGKC |
| pPL N4/9 K36/40 | MDSNGSSQNGSRLLLLLVVSNLLLCQGVVS TPVCPKGPGKC |

**B**

| | | | | | | | | | | | | | translocation efficiency in the absence of TRAM (in %) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PK-RM | - | + | | | | | - | + | | | | | | |
| SR | | | - | + | + | + | | | - | + | + | + | | |
| Sec61 | | | + | - | + | + | | | + | - | + | + | | |
| TRAM | | | + | + | - | + | | | + | + | - | + | | |
| Prot.K | - | - | - | - | - | - | + | + | + | + | + | + | | |
| pPL WT / PL WT | | | | | | | | | | | | | 77 % +/- 11 | A |
| pPL Δ2-11 / PL | | | | | | | | | | | | | 18 % +/- 5 | B |
| pPL N4/9 / PL | | | | | | | | | | | | | 77 % +/- 11 | C |
| pPL K36/40 / PL K36/40 | | | | | | | | | | | | | 83 % +/- 9 | D |
| pPL N4/9 K36/40 / PL K36/40 | | | | | | | | | | | | | 84 % +/- 7 | E |

1 2 3 4 5 6 7 8 9 10 11 12

*Figure 4.* The N-domain of the signal sequence affects TRAM dependence. (*A*) Structure of the signal sequence of wild-type preprolactin (*pPL*) and of signal-sequence mutants. For the wild-type protein, the hydrophobic core of the signal sequence is underlined and charged amino acids are highlighted. The deletion made for mutant pPL Δ2-11 is indicated by a dotted line, and the amino acid changes made in the other mutants are indicated in bold face. Arrow denotes the signal sequence cleavage site. (*B*) Wild-type pPL and pPL signal sequence mutants were tested for translocation into native microsomes (*PK-RM*) or proteoliposomes as described in Fig. 1. Lanes *1–6* show the total products, lanes *7–12* the translocated material protected against digestion by proteinase K (*Prot.K*). The bands of higher mobility seen in lane *12* are prolactin (*PL*) and prolactin K36/40 produced by signal peptidase that contaminates the TRAM preparation. Translocation efficiencies in the absence of TRAM were determined as described in Fig. 1 from 5–10 experiments.

**A**

signal sequence cleavage

| | |
|---|---|
| pPL N4/9 | MDSNGSSQNGSRLLLLLVVSNLLLCQGVVS ↓TPVCPNGPGNC... |
| pPL Δ2-3 | M.....NGSSQNGSRLLLLLVVSNLLLCQGVVS TPVCPNGPGNC |
| pPL Δ2-5 | M.........SSQNGSRLLLLLVVSNLLLCQGVVS TPVCPNGPGNC |
| pPL Δ2-7 | M.............QNGSRLLLLLVVSNLLLCQGVVS TPVCPNGPGNC |
| pPL Δ2-9 | M..................GSRLLLLLVVSNLLLCQGVVS TPVCPNGPGNC |
| pPL Δ2-11 | M....................RLLLLLVVSNLLLCQGVVS TPVCPNGPGNC |
| pPL Δ2-12 | M.....................LLLLLVVSNLLLCQGVVS TPVCPNGPGNC |

**B**

| | | | | | | | | | | | | | translocation efficiency in the absence of TRAM (in %) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PK-RM | - | + | | | | | - | + | | | | | |
| SR | | | - | + | + | + | | | - | + | + | + | |
| Sec61 | | | + | - | + | + | | | + | - | + | + | |
| TRAM | | | + | + | - | + | | | + | + | - | + | |
| Prot.K | - | - | - | - | - | - | + | + | + | + | + | + | |
| pPL N4/9 / PL | | | | | | | | | | | | | 77 % +/- 11 |
| pPL Δ2-3 / PL | | | | | | | | | | | | | 83 % +/- 5 |
| pPL Δ2-5 / PL | | | | | | | | | | | | | 87 % +/- 10 |
| pPL Δ2-7 / PL | | | | | | | | | | | | | 58 % +/- 10 |
| pPL Δ2-9 / PL | | | | | | | | | | | | | 37 % +/- 11 |
| pPL Δ2-11 / PL | | | | | | | | | | | | | 18 % +/- 5 |
| pPL Δ2-12 / PL | | | | | | | | | | | | | 32 % +/- 9 |

1 2 3 4 5 6 7 8 9 10 11 12

*Figure 5.* Gradual shortening of the N-domain of the signal sequence of pPL increases TRAM dependence. (*A*) Structure of the signal sequence of the mutant pPL N4/9 and of its deletion mutants. The hydrophobic core is underlined and the cleavage site is denoted by an arrow. Deletions are indicated by dotted lines. (*B*) The translocation of the proteins into native microsomes (*PK-RM*) or proteoliposomes was tested as described in Fig. 1. *Prot.K*, proteinase K; *PL*, prolactin. The translocation efficiencies for proteoliposomes lacking TRAM were determined from five experiments.

possess any positive charges in its N-domain (Fig. 3). Thus, it appears that the length of the N-domain, but not the number of positive charges contained in it, are determinants of TRAM independence of a secretory protein.

To confirm this conclusion, we produced signal sequence mutants of preprolactin that lacked increasing numbers of amino acids from the N-domain (from 2 to 11 residues; see Fig. 5 *A*). To exclude any interfering side effects of the positive charges, we used the mutant pPL N4/9 as a starting point for the deletion analysis. A deletion of up to 4 residues had little effect on the TRAM independence of the translocation reaction but further deletions resulted in a more pronounced influence of TRAM (Fig. 5 *B*). Surprisingly, removal of the last residue of the N-domain decreased again slightly the TRAM dependence. In contrast to TRAM-dependent wild-type proteins (see Fig. 1), transport in the absence of TRAM was still detectable with all constructs, suggesting that other domains of the signal sequence may also determine TRAM independence.

Since the average hydrophobic core of TRAM-dependent proteins was predicted to be shorter than that of TRAM-independent proteins, we produced several signal sequence mutants of preprolactin with deletions in the hydrophobic core (Fig. 6 *A*). Since the deletion of leucines
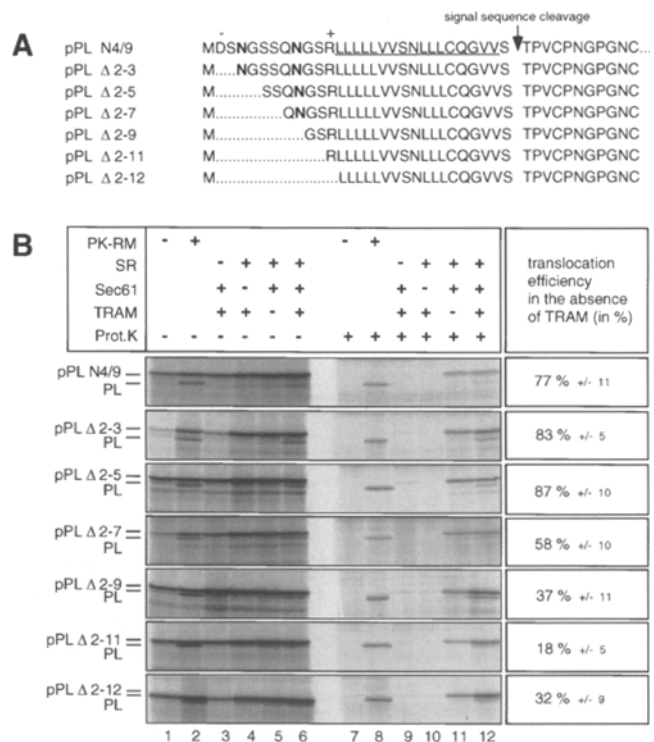
resulted in severe translocation defects even in the presence of TRAM (Jungnickel and Rapoport, 1995; and data not shown), two mutants were constructed that had deletions in a more hydrophilic portion of the signal sequence (pPL ΔVSN, carrying a deletion of residues 19 to 21, and pPL ΔSN, carrying a deletion of residues 20 and 21). These mutants were not affected in their overall transport efficiency (Fig. 6 *B*, lane *8*) but showed a reduced transport efficiency with proteoliposomes that lacked TRAM (lane *11* vs. *12*). It therefore appears that the structure of the hydrophobic core region does have a moderate influence on the TRAM requirement, although more specific effects of the mutations cannot be excluded.

Taken together, the results indicate that TRAM independence is not determined by a single feature of the signal sequence but rather by a combination of several structural characteristcs, such as the length of its hydrophilic, NH₂-terminal domain and the structure of its hydrophobic core.

### TRAM Is Required for the Protease-resistant Insertion of Nascent Chains into the Translocation Site

At which step of the transport process is TRAM required? To address this question, early translocation intermediates of the TRAM-dependent VSV G were analyzed. We first
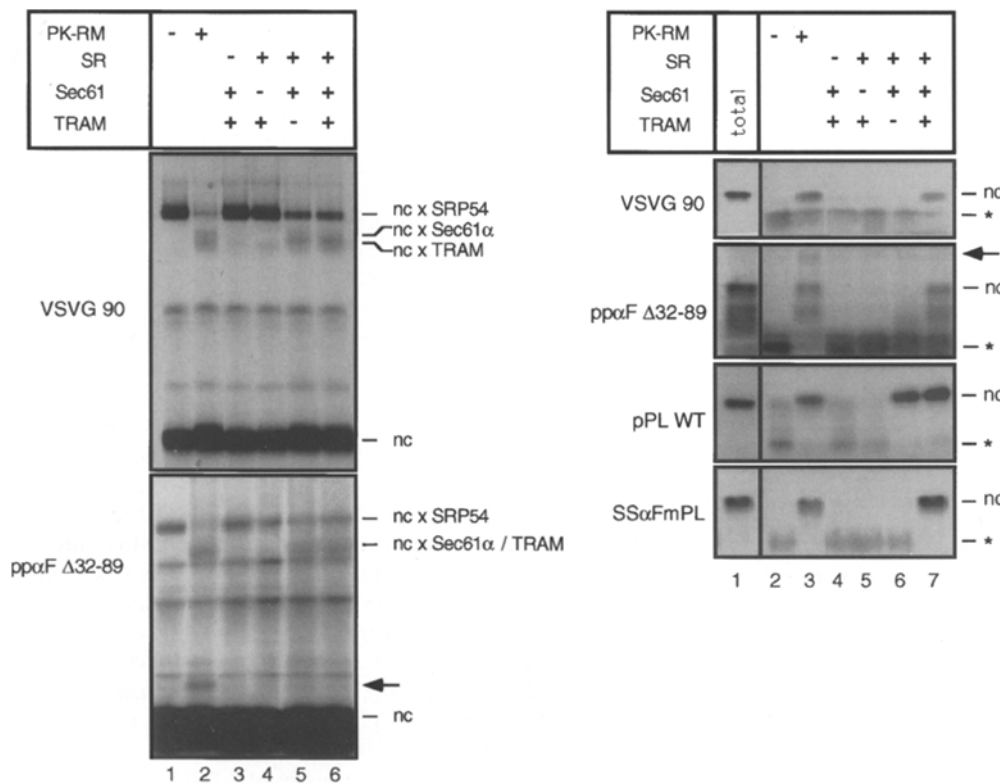
# A

Figure 6. Mutations in the hydrophobic core of the signal sequence of preprolactin affect TRAM dependence. (A) Structure of the signal sequence of preprolactin and of the mutants that carry deletions in the hydrophobic core. Deletions are indicated by dotted lines. (B) The translocation of the proteins was tested as described in Fig. 1. Prot.K, proteinase K; PL, prolactin. The transport efficiencies in the absence of TRAM were determined from 5–8 experiments.

used photo–cross-linking to test whether TRAM is required for the transfer of the nascent chain from SRP into the membrane. A ribosome-bound nascent chain, comprising the first 90 residues of the protein (VSV G90), was synthesized in a wheat germ translation system in the presence of SRP and diazirino-benzoyl-lysyl-tRNA (Kurzchalia et al., 1986; Wiedmann et al., 1987). Addition of the latter results in the incorporation of photoreactive lysyl derivatives at positions of the nascent chain where normally lysines would occur (positions 2, 17, 27, 31, and 59 of the VSV G protein). When the ribosome-nascent chain complex was irradiated in the absence of membranes, a prominent cross-linked product of ∼65 kD was observed (Fig. 7 A, top panel, lane 1) which contained the 54-kD subunit of SRP (as demonstrated by immunoprecipitation; not shown). When microsomal membranes (PK-RM) were added prior to irradiation, the cross-links to SRP54 disappeared and cross-links to the α subunit of the Sec61p complex (Sec61α) and to TRAM could be observed instead (immunoprecipitations not shown), indicating that the nascent chain has been transferred from SRP into the membrane (see Görlich et al., 1992a,b; Jungnickel and Rapoport, 1995). We next used the cross-linking approach to study the binding of VSV G90 to reconstituted proteoliposomes containing purified translocation components. With proteoliposomes lacking the SRP receptor (Fig. 7 A, top panel, lane 3) or the Sec61p complex (lane 4), the intensity of the cross-links to SRP54 did not diminish significantly nor did prominent cross-links to membrane proteins appear, indicating that efficient release of the nascent chain from SRP requires the presence of both membrane proteins, as noted earlier for preprolactin (Görlich and Rapoport, 1993; Jungnickel and Rapoport, 1995). In the presence of both the SRP receptor and Sec61p complex, efficient release of the nascent chain from SRP occurred and cross-links to Sec61α could be detected (lane 5). The additional presence of TRAM had no further effect on the efficiency of

the SRP release (lane 6). Thus, targeting of the TRAM-dependent VSV G protein to the translocation site can occur in the absence of TRAM.

To investigate the possible influence of TRAM on the next step of the translocation process, we made use of a protease-protection assay: if a nascent chain is firmly inserted into the translocation site, it becomes resistant to attack by proteases, presumably because it is buried inside a contiguous channel formed by the ribosome and the Sec61p complex (Connolly et al., 1989; Jungnickel and Rapoport, 1995). If ribosome-nascent chain complexes containing the short fragment of VSV G protein (VSV G90) were incubated with protease in the absence of membranes, a fragment of ∼30 amino acids was produced (Fig. 7 B, top panel, lane 2, asterisk). It most likely represents the COOH-terminal portion of the nascent chain buried inside the ribosome. After incubation with microsomal membranes, the complete chain of 90 amino acids was protected from proteolytic degradation (lane 3). When reconstituted proteoliposomes were used, complete protection was only observed if they contained the SRP receptor, the Sec61p complex and the TRAM protein (lane 7). No protection was seen when TRAM was omitted (lane 6) although the cross-linking experiments had shown that the nascent chain was targeted to the membrane (Fig. 7 A). We therefore conclude that for nascent VSV G polypeptides the presence of TRAM is critical for the next step of the translocation process, their tight insertion into the translocation site.

To confirm this result with another TRAM-dependent protein, we used a mutant of ppαF that lacks a portion of the proregion (ppαFΔ32-89; Rothblatt et al., 1987); the wild-type protein does not contain suitably located lysines and methionines for the incorporation of photoreactive probes and radioactivity, respectively. A polypeptide fragment of 102 amino acids was synthesized in the wheat germ system and used for both cross-linking and protease-protection experiments (Fig. 7 A, bottom panel and B, second panel, respectively). As observed before for the VSV G protein, cross-links to membrane proteins appeared even in the absence of TRAM (Fig. 7 A, bottom panel, lane 5 vs. 6), whereas protease protection of the nascent chain was only seen in its presence (Fig. 7 B, second panel, lanes 6 vs. 7).

In contrast to the behavior of the two TRAM-dependent proteins tested, tight insertion of the TRAM-independent protein preprolactin did not require TRAM (Fig. 7 B, third panel) but only the SRP receptor and the Sec61p complex, as noted earlier (Jungnickel and Rapoport, 1995). Thus, the different behavior of TRAM-dependent and -independent proteins in overall translocation is reflected in their distinct requirements for protease-resistant insertion into the membrane. To confirm this conclusion, we used the TRAM-dependent hybrid protein SSαFmPL that differs from preprolactin only in its signal sequence (see Fig. 2 A). Protease-resistant membrane insertion of a fragment corresponding to the same COOH-terminal truncation was only observed if the TRAM protein was present (Fig. 7 B, bottom panel, lane 6 vs. 7). Thus, the signal sequence determines whether or not TRAM is required for the critical translocation phase of protease-resistant membrane insertion of a polypeptide chain.

To exclude effects of TRAM on earlier translocation

*Figure 7.* TRAM is required for the insertion of TRAM-dependent polypeptide chains into the translocation site. (*A*) The transfer of the nascent chain from SRP into the membrane was followed by photo–cross-linking. A 90–amino acids fragment of the TRAM-dependent VSV G protein (*VSVG 90*) and a fragment of 102 amino acids of a ppαF deletion mutant (*ppαF Δ32-89*) were synthesized in the wheat germ system in the presence of SRP, [$^{35}$S-]methionine, and modified lysyl-tRNA that carries a photoreactive group in the side chain of the amino acid. Aliquots were incubated in the absence or presence of ribosome-stripped microsomes (*PK-RM*) or of proteoliposomes containing different combinations of the SRP receptor (*SR*), the Sec61p complex (*Sec61*), and the TRAM protein (*TRAM*), as indicated. After irradiation, the samples were analyzed by SDS-PAGE and autoradiography. The positions of the non-cross-linked nascent chains (*nc*) and of their cross-linked products containing either the 54-kD subunit of SRP (*SRP54*), the α subunit of the Sec61p complex (*Sec61α*) or the TRAM protein are indicated. The glycosylated form of ppαFΔ32-89 is indicated by an arrow. (*B*) The transfer of the nascent chain into the translocation site was followed by a protease protection assay. VSVG 90 and ppαF Δ32–89 were synthesized in the wheat germ system in the presence of SRP and [$^{35}$S-]methionine, equal aliquots were incubated with microsomes or proteoliposomes, as indicated, and treated with proteinase K. The positions in the SDS gel of the nascent chain (*nc*), of the ribosome-protected fragment of about 30 residues (*asterisk*) and of the glycosylated form of the ppαF mutant (*arrow*) are indicated. The sample shown in lane *1* corresponds to the original aliquot (total) before addition of the protease. Similar experiments were carried out with a fragment of preprolactin containing the first 86 amino acids (*pPL WT*), and with a fragment of the hybrid protein SSαFmPL that contains the signal sequence of prepro-α-factor linked to the mature region of preprolactin and that was truncated at the same COOH-terminal position as pPL WT.

phases which may indirectly influence the insertion of the polypeptide chain into the translocation site, we used a recently established SRP-independent targeting system (Jungnickel and Rapoport, 1995). Ribosome-nascent chain complexes of pPL and SSαFmPL were purified by sedimentation through a high salt cushion, incubated with puromycin/high salt-stripped microsomes (PK-RM) or with proteoliposomes, and submitted to the protease-protection assay (Fig. 8). As reported before for wild-type preprolactin chains (Jungnickel and Rapoport, 1995), under these conditions neither SRP nor SRP receptor are required for the insertion of the nascent chain into the translocation site; the Sec61p complex alone in the lipid bilayer is sufficient (*top panel*, lane *3*). TRAM had only a slight stimulatory effect (lane *3* vs. *5*). If the hybrid protein SSαFmPL was used, protection of the entire nascent chain against proteolytic attack required, in addition to the Sec61p complex, the presence of the TRAM protein in the membrane. In the absence of TRAM, an intermediate-sized fragment was produced that may be similar to one formed from preprolactin carrying a nonfunctional signal sequence (pPLΔ13-15; *bottom panel*, lanes *3*, *5*, and *6*). In the latter case, however, the intermediate was also seen

with TRAM-containing vesicles (lane *3* vs. *5*, *double asterisks*). It thus appears that the translocation of a TRAM-dependent protein is blocked in the absence of TRAM at the same point at which the process would be blocked with a nonfunctional signal sequence. In the case of a TRAM-dependent signal sequence, the block can be overcome by the presence of TRAM to insert the nascent chain into the translocation site of the ER in a protease-resistant manner. In the case of a nonfunctional signal sequence, translocation cannot proceed even in the presence of TRAM.

## Discussion

In this study, we have used a recently established reconstituted system to address the function of TRAM, the component of the mammalian ER translocation apparatus that is least understood. Several conclusions can be drawn from the results: (*a*) the majority of secretory proteins, but not all, require the TRAM protein for their translocation across the membrane; (*b*) the difference between TRAM-dependent and -independent proteins is caused by differences in the structure of their signal sequences; (*c*) several features seem to be required to make a signal sequence
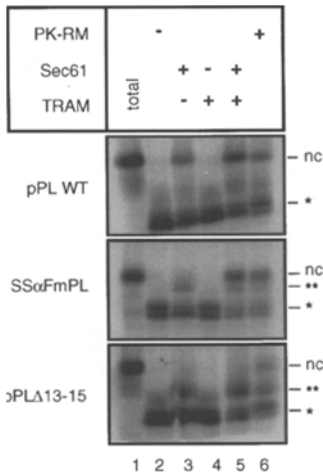
*Figure 8.* TRAM is required for the recognition of TRAM-dependent signal sequences. A protease protection assay was used to test the SRP-independent insertion of short nascent chains into the translocation site. Fragments of preprolactin of 86 amino acids (*pPL WT*) or of the hybrid protein ssαFmPL (containing the signal sequence of ppαF linked to the mature region of pPL) or of the signal sequence mutant pPL Δ13-15 (carrying a deletion of three amino acids in the hydrophobic core) were employed. The latter two proteins were truncated at the same COOH-terminal position as pPL WT. All fragments were synthesized in the wheat germ system and the ribosome-nascent chain complexes were isolated by sedimentation through a sucrose cushion containing a high salt concentration. Aliquots were incubated with microsomes (*PK-RM*) or proteoliposomes and treated with proteinase K. The sample shown in lane *1* corresponds to the original aliquot (total) before addition of the protease. The positions in the SDS gel of the nascent chain (*nc*) and of the ribosome-protected fragment of about 30 residues (*asterisk*) are indicated. The band indicated by two asterisks is a protease-protected fragment of ~50 residues that corresponds to an intermediate of membrane insertion (see Jungnickel and Rapoport, 1995).

TRAM independent, in particular a long NH$_2$-terminal region and perhaps a long hydrophobic core; and (*d*) when TRAM is required for the translocation of a protein, it is needed for the insertion of the nascent chain into the translocation site to reach a protease-resistant state, and not for the preceding targeting step.

These results provide further evidence that the three translocation components, SRP receptor, Sec61p complex, and TRAM constitute a minimum translocation apparatus. Considering the total number of secretory and membrane proteins tested to date (17), there is little doubt that the majority of natural translocation substrates are transported into proteoliposomes containing only these three components. However, some mutant proteins seem to be exceptions. A signal sequence mutant of preprolactin is still inserted to some extent into the translocation site of native microsomes, but not into that of reconstituted proteoliposomes (Jungnickel and Rapoport, 1995). Also, a chimeric ppαF containing the first 12 amino acids of the preprolactin signal sequence in front of its third amino acid, was transported into native microsomes but not into proteoliposomes (data not shown). Together with the low efficiency of transport observed with some wild-type proteins, these data suggest that there may be additional factors that stimulate the translocation process.

Although we have divided proteins into TRAM-dependent and -independent classes, the distinction is clearly not absolute: the translocation of so-called TRAM-independent proteins is somewhat stimulated by the presence of TRAM in the membrane, and TRAM-dependent proteins sometimes exhibit a low level of translocation in the absence of TRAM. Nevertheless, for naturally occurring proteins the classification is quite straightforward. In the case of proteins with mutagenized signal sequences, however, intermediary degrees of TRAM dependence were seen, and we have not been able to completely convert by mutagenesis a TRAM-independent signal sequence into a TRAM-dependent one. Most likely, several structural features of the signal sequence must come together to produce almost complete TRAM independence. Synergistic effects of the structures of the NH$_2$-terminal region and of the hydrophobic core have been observed before: a critical balance between them determines whether a hydrophobic segment functions as a cleavable signal sequence or a signal-anchor sequence, and whether the latter has its NH$_2$ terminus in- or outside the cytoplasm (Sakaguchi et al., 1992). Unfortunately, our choices of signal sequence mutations were severely restricted by the fact that in cases where translocation in the presence of TRAM is much reduced, a conclusion concerning the function of this component could not be drawn. Even though our experiments demonstrate a crucial role for the signal sequence in determining TRAM dependence, there may be cases where the mature region of a polypeptide chain has an influence, because TRAM functions before the signal sequence is cleaved.

The translocation phase during which TRAM is required for TRAM-dependent proteins is a critical step inside the membrane in which the nascent chain is inserted into the translocation site to reach a protease-protected state. Previous experiments with the TRAM-independent protein preprolactin have shown that during this step the signal sequence is recognized in a process that only requires the Sec61p complex in the phospholipid bilayer (Jungnickel and Rapoport, 1995). Our present data show that for TRAM-dependent proteins, the same step also requires the TRAM protein. In the absence of TRAM, the translocation process seems to be aborted at a point at which the ribosome-nascent chain is only weakly bound to the membrane, the same point at which translocation is blocked for nascent chains containing a nonfunctional signal sequence (Jungnickel and Rapoport, 1995). Together with the fact that TRAM dependence is determined by the structure of the signal sequence, it therefore appears that in these cases the TRAM protein is required for signal sequence recognition in the membrane. It remains to be determined, however, whether the signal sequence is recognized by a protein–protein interaction involving the Sec61p complex alone or in conjunction with TRAM, or whether it first partitions into the phospholipid bilayer and then enters the translocation channel laterally. Eventually, upon stable insertion into the translocation site, the signal sequence can be cross-linked to Sec61α, TRAM, and lipids (High et al., 1993; Mothes et al., 1994; Martoglio et al., 1995). Since TRAM is found in proximity of short nascent chains of the TRAM-independent protein preprolactin (Görlich et al., 1992a), it appears that TRAM is part of the translocation site into which polypeptide chains are inserted, and that it therefore participates in the translocation of every protein, irrespective of whether it is actually required.

The precise molecular function of the TRAM protein

remains unclear but several possibilities may be considered. When a nascent chain is entering the translocation site, it forms presumably a loop structure, with one side of the hairpin formed by the signal sequence (Shaw et al., 1988; Mothes et al., 1994). In one model, TRAM would help to retain the $NH_2$ terminus of the signal sequence on the cytoplasmic side of the membrane, preventing its flipping through the membrane. Such a model would be consistent with the observation that TRAM contacts mostly the polar, $NH_2$-terminal region of the signal sequence of preprolactin (High et al., 1993; Mothes et al., 1994). In TRAM-independent proteins this polar region is generally longer which might keep this domain in the cytosol and prevent its spontaneous flipping through the membrane even if TRAM is absent. Consistent with this idea, our results show that a protein becomes more dependent on TRAM if this $NH_2$-terminal region is gradually shortened. However, it seems unlikely that TRAM exclusively binds to the $NH_2$-terminal region since the latter is very short for some TRAM-dependent proteins. Also, the fact that TRAM is a multispanning membrane protein is more consistent with the notion that it has a function inside the membrane, rather than only at its cytoplasmic side.

An alternative model is that TRAM guides a hydrophobic signal sequence into the membrane. This might be achieved by an interaction with the hydrophobic core of the signal sequence or by a local perturbation of the lipid bilayer. According to this model, in the absence of TRAM only those signal sequences would be able to enter the translocation site that can partition into the lipid sufficiently well on their own. Indeed, TRAM-independent signal sequences tend to have longer hydrophobic cores, and perturbations in this region render the protein more TRAM dependent. However, TRAM dependence has also been observed for signal-anchor type membrane proteins whose signal sequence is clearly very long and hydrophobic and can serve to stably anchor them in the lipid bilayer (Görlich and Rapoport, 1993). One would therefore have to assume that it is the transfer into the lipid, rather than the final, membrane-inserted state, that is facilitated by TRAM. Considering that the transfer process requires both the passage through the region of charged head groups of the phospholipid bilayer and the penetration into the hydrophobic region of the membrane, it may not be surprising that it is not only hydrophobicity of the signal sequence that determines TRAM dependence. In either model, the TRAM protein could also act indirectly by binding to the Sec61p complex and stabilizing or activating it to cope with certain signal sequences.

Whether TRAM plays a role beyond the initiation of the translocation process is not yet clear. Cross-linking experiments with preprolactin have shown that TRAM is no longer in proximity of the nascent chain once the signal sequence has been cleaved off (Mothes et al., 1994). However, we consider it possible that it remains in the translocation site, though shielded from the nascent chain. This would explain why TRAM is as abundant as the Sec61p complex (Görlich et al., 1992a; Görlich and Rapoport, 1993) while the SRP receptor, which is only involved in the initiation process, is present in distinctly lower amounts (Tajima et al., 1986). While the precise role of TRAM needs to be further defined, the present results demonstrate that it is an important component of the translocation machinery of the mammalian ER membrane.

*References*

Connolly, T., P. Collins, and R. Gilmore. 1989. Access of proteinase K to partially translocated nascent polypeptides in intact and detergent-solubilized membranes. *J. Cell Biol.* 108:299–307.

Crowley, K.S., G.D. Reinhart, and A.E. Johnson. 1993. The signal sequence moves through a ribosomal tunnel into a noncytoplasmic aqueous environment at the ER membrane early in translocation. *Cell.* 73:1101–1115.

Crowley, K.S., S.R. Liao, V.E. Worrell, G.D. Reinhart, and A.E. Johnson. 1994. Secretory proteins move through the endoplasmic reticulum membrane via an aqueous, gated pore. *Cell.* 78:461–471.

Gilmore, R., P. Walter, and G. Blobel. 1982. Protein translocation across the endoplasmic reticulum. II. Isolation and characterization of the signal recognition particle receptor. *J. Cell Biol.* 95:470–477.

Görlich, D., and T.A. Rapoport. 1993. Protein translocation into proteoliposomes reconstituted from purified components of the endoplasmic reticulum membrane. *Cell.* 75:615–630.

Görlich, D., E. Hartmann, S. Prehn, and T.A. Rapoport. 1992a. A protein of the endoplasmic reticulum involved early in polypeptide translocation. *Nature (Lond.).* 357:47–52.

Görlich, D., S. Prehn, E. Hartmann, K.-U. Kalies, and T.A. Rapoport. 1992b. A mammalian homolog of Sec61p and SecYp is associated with ribosomes and nascent polypeptides during translocation. *Cell.* 71:489–503.

High, S., B. Martoglio, D. Görlich, S.S.L. Andersen, A.J. Ashford, A. Giner, E. Hartmann, S. Prehn, T.A. Rapoport, B. Dobberstein et al. 1993. Site-specific photocross-linking reveals that Sec61p and TRAM contact different regions of a membrane-inserted signal sequence. *J. Biol. Chem.* 268:26745–26751.

Jungnickel, B., and T.A. Rapoport. 1995. A posttargeting signal sequence recognition event in the endoplasmic reticulum membrane. *Cell.* 82:261–270.

Kalies, K.-U., D. Görlich, and T.A. Rapoport. 1994. Binding of ribosomes to the rough endoplasmic reticulum mediated by the Sec61p-complex. *J. Cell Biol.* 126:925–934.

Kurzchalia, T.V., M. Wiedmann, A.S. Girshovich, E.S. Bochkareva, H. Bielka, and T.A. Rapoport. 1986. The signal sequence of nascent preprolactin interacts with the 54K polypeptide of the signal recognition particle. *Nature (Lond.).* 320:634–636.

Lauring, B., H. Sakai, G. Kreibich, and M. Wiedmann. 1995a. Nascent polypeptide-associated complex protein prevents mistargeting of nascent chains to the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA.* 92:5411–5415.

Lauring, B., G. Kreibich, and M. Wiedmann. 1995b. The intrinsic ability of ribosomes to bind to endoplasmic reticulum membranes is regulated by signal recognition particle and nascent-polypeptide-associated complex. *Proc. Natl. Acad. Sci. USA.* 92:9435–9439.

Martoglio, B., M.W. Hofmann, J. Brunner, and B. Dobberstein. 1995. The protein-conducting channel in the membrane of the endoplasmic reticulum is open laterally toward the lipid bilayer. *Cell.* 81:207–214.

Meyer, D.I., E. Krause, and B. Dobberstein. 1982. Secretory protein translocation across membranes—the role of the docking protein. *Nature (Lond.).* 297:647–650.

Mothes, W., S. Prehn, and T.A. Rapoport. 1994. Systematic probing of the environment of a translocating secretory protein during translocation through the ER membrane. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:3937–3982.

Oliver, J., B. Jungnickel, D. Görlich, T.A. Rapoport, and S. High. 1995. The Sec61p complex is essential for the insertion of protein into the membrane of the endoplasmic reticulum. *FEBS Lett.* 362:126–130.

Rapoport, T.A. 1992. Transport of proteins across the endoplasmic reticulum membrane. *Science (Wash. DC).* 258:931–936.

Rothblatt, J.A., J.R Webb, G. Ammerer, and D.I. Meyer. 1987. Secretion in yeast: structural features influencing the posttranslational translocation of prepro-α-factor *in vitro. EMBO (Eur. Mol. Biol. Organ.) J.* 6:3455–3463.

Sakaguchi, M., R. Tomiyoshi, T. Kuroiwa, K. Mihara, and T. Omura. 1992. Functions of signal and signal-anchor sequences are determined by the balance between the hydrophobic segment and the $NH_2$-terminal charge. *Proc. Natl. Acad. Sci. USA.* 89:16–19.

Shaw, A.S., P.J. Rottier, and J.K. Rose. 1988. Evidence for the loop model of signal-sequence insertion into the endoplasmic reticulum. *Proc. Natl. Acad.*

*Sci. USA.* 85:7592–7596.

Simon, S.M., and G. Blobel. 1991. A protein-conducting channel in the endoplasmic reticulum. *Cell.* 65:371–380.

Tajima, S., L. Lauffer, V.L. Rath, and P. Walter. 1986. The signal recognition particle receptor is a complex that contains two distinct polypeptide chains. *J. Cell Biol.* 103:1167–1178.

von Heijne, G. 1985. Signal sequences. The limits of variation. *J. Mol. Biol.* 184: 99–105.

Walter, P., and G. Blobel. 1983. Preparation of microsomal membranes for cotranslational protein translocation. *Methods Enzymol.* 96:84–93.

Walter, P., and A.E. Johnson. 1994. Signal sequence recognition and protein targeting to the endoplasmic reticulum membrane. *Annu. Rev. Cell Biol.* 10:87–119.

Walter, P., I. Ibrahimi, and G. Blobel. 1981. Translocation of proteins across the endoplasmic reticulum. I. Signal recognition protein (SRP) binds to in vitro assembled polysomes synthesizing secretory protein. *J. Cell Biol.* 91:545–550.

Wiedmann, M., T.V. Kurzchalia, H. Bielka, and T.A. Rapoport. 1987. Direct probing of the interaction between the signal sequence of nascent preprolactin and the signal recognition particle by specific cross-linking. *J. Cell Biol.* 104:201–208.

Wiedmann, B., H. Sakai, T.A. Davis, and M. Wiedmann. 1994. A protein complex required for signal-sequence-specific sorting and translocation. *Nature (Lond.).* 370:434–440.