

Unassisted translocation of large polypeptide domains across phospholipid bilayers

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Although transmembrane proteins generally require membrane-embedded machinery for integration, a few can insert spontaneously into liposomes. Previously, we established that the tail-anchored (TA) protein cytochrome b(5) (b5) can post-translationally translocate 28 residues downstream to its transmembrane domain (TMD) across protein-free bilayers (Brambillasca, S., M. Yabal, P. Soffientini, S. Stefanovic, M. Makarow, R.S. Hegde, and N. Borgese. 2005. *EMBO J.* 24:2533–2542). In the present study, we investigated the limits of this unassisted translocation and report that surprisingly long (85 residues) domains of different sequence and charge placed downstream of b5's TMD

can posttranslationally translocate into mammalian microsomes and liposomes at nanomolar nucleotide concentrations. Furthermore, integration of these constructs occurred in vivo in translocon-defective yeast strains. Unassisted translocation was not unique to b5 but was also observed for another TA protein (protein tyrosine phosphatase 1B) whose TMD, like the one of b5, is only moderately hydrophobic. In contrast, more hydrophobic TMDs, like synaptobrevin's, were incapable of supporting unassisted integration, possibly because of their tendency to aggregate in aqueous solution. Our data resolve long-standing discrepancies on TA protein insertion and are relevant to membrane evolution, biogenesis, and physiology.

Introduction

Insertion of transmembrane proteins into biological membranes generally requires the activity of protein-conducting channels that affect translocation of the exoplasmic polar domains and integration into the bilayer of the membrane-spanning segments of the nascent membrane protein (Rehling et al., 2004; Osborne et al., 2005). Because the translocation channels themselves are membrane embedded, one might expect them to rely on copies of themselves for proper integration, and this indeed seems to be the case for the core translocation channels of the outer mitochondrial membrane (TOM40; Rapaport and Neupert, 1999) and of the ER (Sec61 α ; Knight and High, 1998). Such a dependency of each newly synthesized component of a given translocation complex on functionally integrated copies of the same translocator may underlie the fidelity of the process of membrane expansion, by which each membrane serves as a template

for its own growth. However, it also poses an evolutionary problem: how did biological membranes initially assemble the minimal membrane-integrated machinery required to permit the insertion of vital functional protein components? One possibility is that primitive membrane proteins can insert into lipid bilayers without assistance from protein-conducting channels. In support of this notion, a few proteins with short exoplasmic domains have been shown to be capable of transmembrane integration into protein-free lipid bilayers (van Dalen and de Kruijff, 2004; Brambillasca et al., 2005). We refer to this type of integration as unassisted, meaning that the insertion does not require membrane proteins whether or not cytosolic chaperones are involved in maintaining the substrate protein in a conformation competent for integration.

Although most of the investigated proteins capable of unassisted integration are prokaryotic (van Dalen and de Kruijff, 2004), at least one eukaryotic protein, the ER form of cytochrome b(5) (b5), inserts very efficiently with transmembrane topology into protein-free liposomes, provided that these have low cholesterol content (Brambillasca et al., 2005). b5 is a member of the group of C-tail-anchored (TA) proteins. Proteins of this diverse group play a variety of fundamental roles in

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Abbreviations used in this paper: b5, cytochrome b(5); CPY, carboxypeptidase Y; PC, phosphatidylcholine; PF, protected fragment; PK, protease K; PTP1B, protein tyrosine phosphatase 1B; RM, rough microsome; SRP, signal recognition particle; Syb, synaptobrevin; TA, tail anchored; TB, translocation buffer; TMD, transmembrane domain; VSVG, vesicular stomatitis virus glycoprotein.

The online version of this article contains supplemental material.

membrane biogenesis and trafficking and are characterized by the presence of an N-terminal cytosolic region and of a single transmembrane domain (TMD) very close to the C terminus (Borgese et al., 2003). To qualify as a true TA protein, the C-terminal polar domain, which is downstream of the TMD, must not exceed 25–30 residues so that during synthesis, the hydrophobic TMD is not exposed to the cytosol until the polypeptide is terminated and released from the ribosome. Therefore, the nascent polypeptide does not have a chance to interact with signal recognition particle (SRP), which mediates cotranslational integration, and insertion into the bilayer must occur posttranslationally. In single-spanning proteins with longer C-terminal polar domains, the TMD becomes available to SRP while still in the nascent chain; these proteins can be cotranslationally integrated into the ER membrane, thus qualifying as bona fide type II proteins (Borgese et al., 2003).

In our previous work, unassisted transbilayer integration was demonstrated for a b5 construct tagged at its C terminus with a 19-residue sequence derived from the N terminus of bovine opsin, which provides an *N*-glycosylation consensus site as well as an epitope (Brambillasca et al., 2005). The addition of this tag brought the length of the C-terminal polar domain to 28 residues, allowing us to monitor its translocation by a protease protection assay (Brambillasca et al., 2005). In the present study, we have investigated the limit of the length of the C-terminal polar domain appended to b5's TMD that can be translocated across protein-free lipid bilayers. Although it is clear that further lengthening of the C-terminal polar domain will result in a type II protein that can potentially use the SRP-dependent cotranslational Sec61-based pathway, our question is focused on the possible existence of an alternative overlapping, unassisted posttranslational pathway. Using *in vitro* and *in vivo* approaches in the mammalian and yeast systems, we show that the TMD of b5 can support the unassisted translocation of surprisingly long and differently charged C-terminal polar sequences with low or no energy requirements.

Although posttranslational integration is a hallmark of TA protein biogenesis, different TA proteins appear to have different requirements for membrane integration. Thus, although b5 undergoes unassisted transbilayer integration, the v-SNARE synaptobrevin 2 (Syb2) requires energy and a proteinaceous factor or factors of the ER membrane (Kutay et al., 1995; Kim et al., 1997; Abell et al., 2004; High and Abell, 2004). Because the unassisted translocation that we observed for our b5 constructs appeared to be relatively insensitive to the sequence of the luminal domain, we investigated whether differences in the TMD underlie the different requirements for TA protein integration. We find that replacement of the b5 TMD with the more hydrophobic one of Syb2 or simply an increase in the hydrophobicity of b5's TMD obtained by a few amino acid substitutions results in constructs that show the same requirements for energy and for an ER proteinaceous component as native Syb. Conversely, a construct carrying a mutated Syb2 TMD with reduced hydrophobicity as well as the TA protein tyrosine phosphatase 1B (PTP1B), whose TMD, like the one of b5, is only moderately hydrophobic, inserts into lipid bilayers without assistance. Our results suggest a mechanism by which primitive

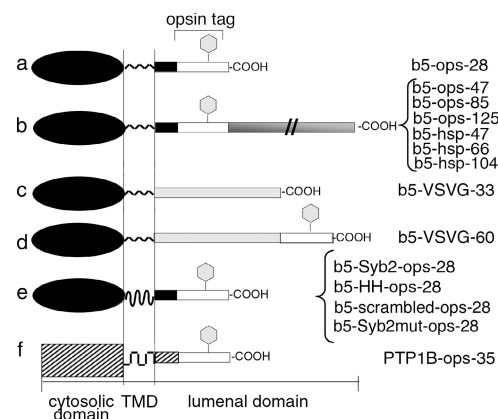


Figure 1. Schematic representation of the constructs used in this study. The opsin tag (corresponding to the first 19 amino acids of the bovine protein) is shown as an open box, with the *N*-glycosylation site represented by a hexagon. The numbers in the name of each construct indicate the total length of the luminal sequence. Sequences deriving from b5 are depicted in black, those from PTP1B are depicted as striped boxes, and those from the cytoplasmic tail of VSVG are in light gray. The shaded gray box in panel b indicates sequences of different lengths deriving from bovine opsin or yeast Hsp150. b5-ops-47 carries the entire N-terminal luminal sequence of bovine opsin, which has been duplicated and triplicated in b5-ops-85 and -125, respectively; Hsp-elongated constructs contain one, two, or four copies of a 19-residue repeated sequence of the yeast protein Hsp150. The constructs illustrated in panels a–d all have the TMD of b5; the constructs illustrated in panel e have an altered TMD. In b5-HH-ops-28, b5's TMD has been mutated to be more hydrophobic, and in b5-scrambled-ops-28, the order of amino acids in b5's TMD has been changed. In b5-Syb2-ops-28 and b5-Syb2mut-ops-28, it has been replaced by the one of Syb2 or by a mutated, less hydrophobic version thereof. The TMDs of b5 (a–d), PTP1B (f), and Syb2 or mutated TMDs of Syb2 and b5 (e) are indicated with different symbols. Sequences of the TMDs and luminal domains are given in Tables I and S1 (available at <http://www.jcb.org/cgi/content/full/jcb.200608101/DC1>), respectively.

membranes may have assembled in the absence of modern translocation machinery and identify TMD hydrophobicity as the feature that determines the different requirements for transmembrane integration of TA proteins.

Results

Size limits for the posttranslational translocation of polar domains appended to b5's C terminus

In previous studies (Pedrazzini et al., 2000; Borgese et al., 2001; Yabal et al., 2003; Brambillasca et al., 2005), we demonstrated the posttranslational transmembrane integration into ER membranes of a b5 variant engineered to contain an *N*-glycosylation site (derived from bovine opsin) near the C terminus (Pedrazzini et al., 2000; and here renamed b5-ops-28). To investigate whether longer C-terminal polar domains can also be translocated in the posttranslational mode, we generated a battery of b5 constructs in which the C-terminal domain was progressively elongated to 125 amino acid residues. These constructs were obtained by fusing unrelated oligopeptides (derived from the N-terminal domain of bovine opsin, from the yeast protein Hsp150, or from the cytosolic domain of vesicular stomatitis virus glycoprotein [VSVG]) downstream of the original construct, b5-ops-28, or immediately after b5's TMD. The constructs were

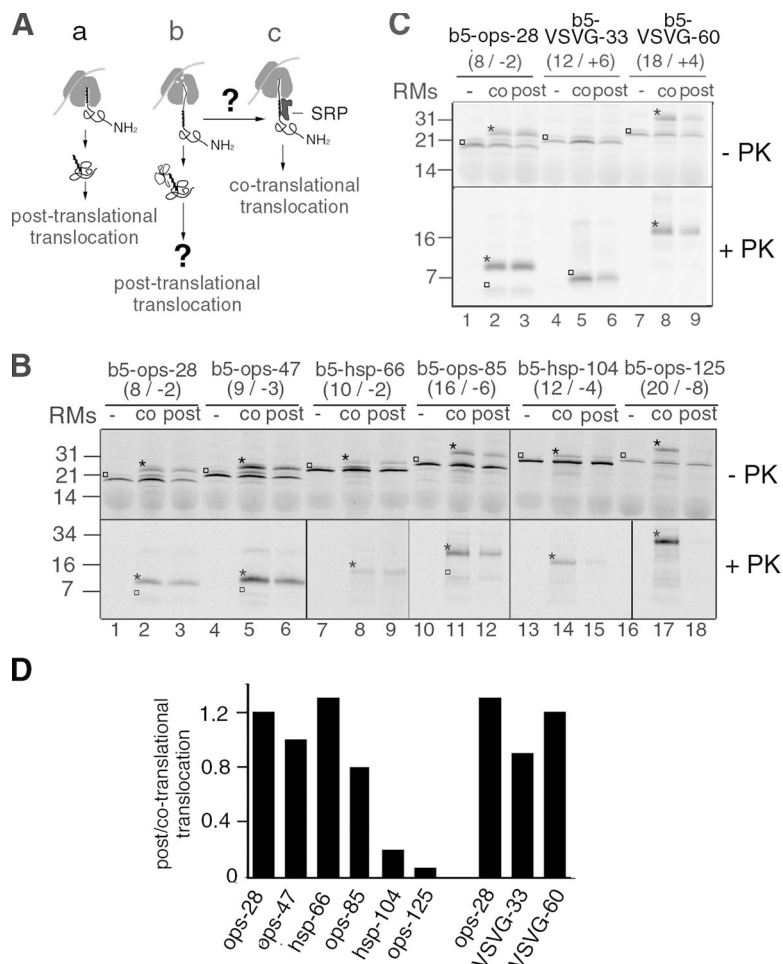


Figure 2. Comparison between co- and posttranslational translocation efficiencies for b5 constructs with extended luminal domains. (A) Illustration of the different hypothetical modes of insertion into RMs for the constructs in this study. The thickened part of the polypeptide chain represents the hydrophobic membrane-anchoring segment (see Results for further explanation). (B) b5-based constructs were incubated with or without RMs after (post) in vitro translation or with RMs during translation (co) as indicated. An aliquot of each sample was directly subjected to SDS-PAGE (top; -PK), whereas the remaining was digested with PK (bottom; +PK). (C) Co- or post-translational translocation reactions for VSVG-elongated constructs. (B and C) Asterisks indicate the glycosylated form of both the full-length proteins (-PK) and the PFs (+PK), whereas the boxes indicate the corresponding nonglycosylated forms. For each construct, the total number of charged amino acids and the net charge of the luminal sequence are reported in parentheses above the lanes. (D) Comparison between efficiencies of post- versus cotranslational translocation for the constructs illustrated in B and C (see Materials and methods).

named according to the origin of the appended sequence and to the length of the entire C-terminal sequence downstream of the TMD (Fig. 1, a–d; and sequences in Table S1, available at <http://www.jcb.org/cgi/content/full/jcb.200608101/DC1>). For these extended constructs, beyond a certain length of the attached sequence, we might expect a shift to the cotranslational SRP-dependent mechanism of translocation because the TMD will emerge from the ribosome before chain termination and be recruited via SRP to the Sec61 translocon, as occurs with classic type II membrane proteins (Fig. 2 A).

To compare posttranslational and cotranslational translocation efficiencies, ER rough microsomes (RMs) were either added during translation of the various constructs or after the termination of protein synthesis and removal of ribosomes (Fig. 2). To follow translocation, we used our previously developed stringent assays (Brambillasca et al., 2005), which are based both on the M_r shift caused by *N*-glycosylation of the translocated opsin sequence and on protection from protease K (PK) digestion of the translocated fragment. An aliquot of each sample was directly analyzed by SDS-PAGE to determine the amount of protein synthesized and the extent of glycosylation (Fig. 2, B and C; top, -PK), whereas the rest was digested with PK and subjected to immunoprecipitation to recover the protected fragment (PF; Fig. 2, B and C; bottom, +PK; see Brambillasca et al., 2005 for a full characterization of the assay).

As shown in Fig. 2 (B and C), when the constructs were not exposed to RMs, only the nonglycosylated protein was detected (boxes indicate the nonglycosylated full-length protein or PF). Likewise, PFs were not recovered from samples incubated in the absence of membranes nor from samples incubated with membranes but digested with PK in the presence of detergent (Fig. S1 A, available at <http://www.jcb.org/cgi/content/full/jcb.200608101/DC1>). Instead, when RMs were present either during or after translation, a portion of each construct with luminal domain up to 85 residues was converted to the glycosylated form (Fig. 2, B and C; asterisks indicate the glycosylated full-length protein or PF) except the construct b5-VSVG-33, which lacks an *N*-glycosylation consensus sequence (Fig. 2 C). In agreement, these constructs, after co- or posttranslational incubation with RMs followed by PK treatment in the absence of detergent, generated a PF, most of which was glycosylated (again, with the exception of b5-VSVG-33). In contrast, the constructs bearing a sequence longer than 100 amino acids downstream of the TMD, although capable of cotranslational translocation, were no longer able to insert posttranslationally, as indicated by the lack of glycosylation and protection from proteolysis (Fig. 2 B, compare lane 14 with 15 and lane 17 with 18).

Quantitative comparison of co- and posttranslational translocation (Fig. 2 D) revealed equal efficiencies for the

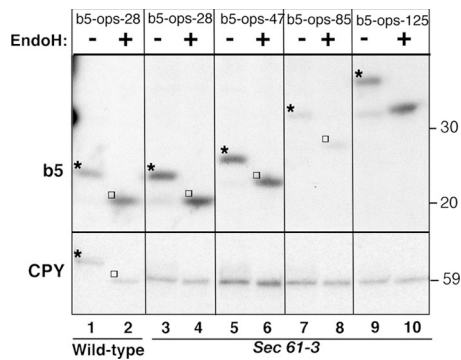


Figure 3. Extended C-terminal domains of b5 constructs are translocated across the ER membrane of yeast mutants defective in translocon function. Wild-type yeast cells (lanes 1 and 2) or yeast cells harboring the *sec61-3* mutation transformed with the indicated b5 construct were incubated in low glucose medium for 1 h at 38°C and were ³⁵S labeled for 5 min at the same temperature. The cells were lysed and subjected to immunoprecipitation with antiopsin antibody (top) or CPY antiserum (bottom). The immunoprecipitates were divided in two, and one part was digested with EndoH before SDS-PAGE analysis as indicated. Asterisks and boxes indicate the glycosylated and nonglycosylated products, respectively.

constructs with C-terminal polar domains of up to 66 residues, whereas the construct with 85 luminal residues showed slightly reduced efficiency, and those with >100 residues showed severely reduced efficiency in the post- versus the cotranslational mode. Thus, remarkably large domains of different sequence and net charge (Fig. 2, B and C; indicated above the lanes) are capable of posttranslational translocation. Interestingly, as previously shown for b5-ops-28 (Brambillasca et al., 2005), only the C terminus and not the N terminus of the recombinant proteins was translocated, as demonstrated by the lack of protection of b5's catalytic domain from PK digestion (unpublished data).

In a previous study, we showed that the transmembrane integration of b5-ops-28 occurs efficiently in yeast mutants that are defective in the function of the translocon or translocon

accessory proteins (Yabal et al., 2003). In this study, we investigated whether the extended constructs could also translocate their C terminus across the ER of yeast *sec61* mutants. As shown in Fig. 3, in the temperature-sensitive *sec61-3* mutant, all of the analyzed recombinant proteins were fully or nearly fully glycosylated at the restrictive temperature, as indicated by the shift in electrophoretic mobility obtained after digestion with endoglycosidase H. In contrast, the glycosylation of carboxypeptidase Y (CPY), a protein that depends on the Sec machinery for its posttranslational translocation, was severely reduced at the restrictive temperature (Fig. 3, bottom), demonstrating inactivation of the mutant Sec61 protein by heat treatment. Similar results were obtained with another temperature-sensitive Sec61p mutant, *sec61-2* (Table S2, available at <http://www.jcb.org/cgi/content/full/jcb.200608101/DC1>; and not depicted). Thus, Sec61-independent translocation in vivo appeared to be even more permissive than posttranslational translocation in vitro because even the b5-ops-125 construct was glycosylated in the yeast mutants.

Unassisted transmembrane integration of extended constructs into protein-free liposomes

In a previous study (Brambillasca et al., 2005), we demonstrated that b5-ops-28 can translocate its C terminus across protein-free phospholipid liposomes as efficiently as across RMs. Therefore, we investigated whether the extended constructs that retain the ability to posttranslationally translocate into RMs were also competent for transmembrane insertion into pure lipid vesicles prepared by the extrusion of a mixture of bovine liver phosphatidylcholine (PC)/phosphatidylethanolamine or PC alone (Fig. 4 A). Insertion into RMs was again assessed by glycosylation of the full-length protein (Fig. 4 A, top) and PF recovery (Fig. 4 A, bottom). In the case of liposomes, translocation of the C terminus cannot be assayed by glycosylation but is revealed by the

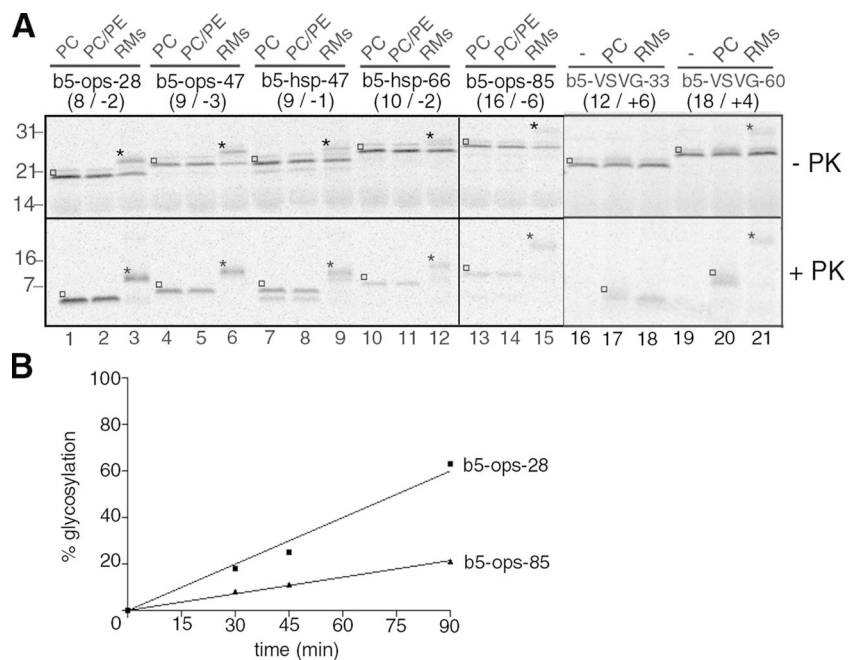


Figure 4. Posttranslational translocation of extended luminal domains of b5 constructs occurs across ER microsomes and protein-free liposomes with equal efficiency. (A) Posttranslational translocation reactions were performed with PC or PC/phosphatidylethanolamine (PE; 4:1 ratio) liposomes, with RMs at equivalent phospholipid concentration (0.7 μg phospholipids/μl) or without added vesicles as indicated. The total number of charged amino acids and the net charge of the luminal sequence are reported in parentheses above the lanes. Asterisks and boxes indicate the glycosylated and nonglycosylated products, respectively. (B) Time course of translocation into RMs of b5-ops-28 and -85 monitored by glycosylation.

protease protection assay (Fig. 4 A; nonglycosylated PFs are indicated by boxes in the bottom panel). Quite remarkably, all C-terminal domains capable of posttranslational translocation across RMs were translocated equally well across protein-free vesicles independently of length, sequence, and charge, and this was true even for the highly charged 85 residues of b5-ops-85 (Fig. 4 A, lanes 13–15). As expected, the 104- and 125-residue-long C-terminal tails were not observed to insert into protein-free liposomes (Fig. S1 B).

Although b5-ops-85 inserts equally well into RMs and liposomes, the extent of its translocation into both of these vesicles was less than that of the parent construct b5-ops-28. This lower efficiency might be the result of either heterogeneity in the translocation competence of the longer construct such that only a fraction of molecules can insert or a reduced translocation rate of equivalent b5-ops-85 molecules. As shown in the time course experiment in Fig. 4 B, the proportion of glycosylated molecules increased linearly with time for both b5-ops-85 and -28 during a period of 90 min, suggesting that *in vitro*-translated b5-ops-85 constitutes a homogeneous population of slowly translocating molecules.

Role of TMD in TA protein translocation

Although b5 efficiently translocates its C terminus across the bilayer without assistance from ER proteins, other TA proteins, like Syb2, require one or more proteinaceous components of the

ER for their integration (High and Abell, 2004). Because the protein-independent mechanism seems to be quite permissive with regard to the C-terminal sequence to be translocated, we investigated the role of the cytosolic and TMD in determining the requirements for TA protein insertion. We excluded the involvement of b5's cytosolic N-terminal domain by replacing this region with GFP in the b5-opsin-85 construct and observing that the resulting protein was still able to efficiently translocate across pure lipid liposomes (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200608101/DC1>). Therefore, we focused on the TMD, replacing the core of b5's TMD with the one of Syb2 (b5-Syb2-ops-28; Fig. 1 e). As shown in Fig. 5 A, the new construct was as efficiently glycosylated and translocated across posttranslationally added RMs as the parent construct b5-ops-28. However, different from b5-ops-28, the construct with Syb2's TMD was unable to translocate its C terminus across protein-free liposomes (Fig. 5 A, bottom). b5-Syb2-ops-28, in contrast to the parent construct, generated two background bands after PK digestion in the absence of vesicles, which comigrated with the glycosylated and nonglycosylated PF (Fig. 5 A, lane 4); as can be seen in Fig. 5 A (lane 6), the intensity of the PF bands obtained after incubation with PC liposomes was similar to that of the background bands.

We next asked whether the different behaviors of the two recombinant proteins was caused by a specific amino acid sequence or, instead, was caused by differences in the

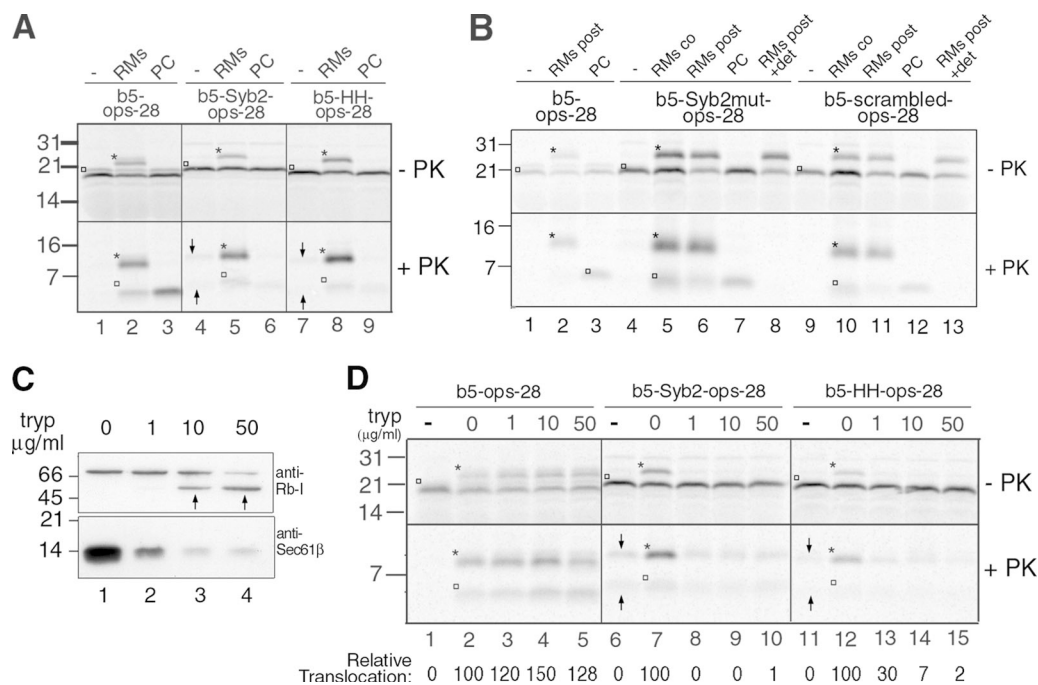


Figure 5. TMDs with increased hydrophobicity require a proteinaceous component of the ER for their insertion. (A and B) Co- or posttranslational translocations were performed with the indicated constructs, without added vesicles, or with RMs or PC liposomes (both at 0.7 µg phospholipids/µl). (A) Background bands generated after PK treatment in the absence of membranes are indicated with arrows (lanes 4 and 7). (B) Detergent controls for b5-Syb2mut-ops28 and b5-scrambled-ops-28 are shown (lanes 7 and 13). For the other constructs, detergent controls are shown in Fig. S1 A (available at <http://www.jcb.org/cgi/content/full/jcb.200608101/DC1>). (C) Analysis of RMs treated with different amounts of trypsin by immunoblotting. Digestion of the cytosolic region of ribophorin I results in a lower M_r band (arrows), which is revealed by the antiribophorin antibody raised against a luminal epitope. (D) After digestion of RMs with trypsin at the indicated concentrations, equal aliquots of the treated vesicles were incubated with the indicated *in vitro*-synthesized proteins. Background bands generated in the absence of vesicles are indicated with arrows. The percentage of translocation efficiency, corrected for background and normalized to the efficiency obtained in mock-treated RMs, is given below the lanes. Asterisks and boxes indicate the glycosylated and nonglycosylated products, respectively.

Table I. Properties of TMDs of the constructs used in this study

Protein	TMD sequence	Mode of calculation ^a	Number of residues in TMD	Hydrophobicity (GES scale)	
				Total	Mean ^c
b5	... DSNSSW WTNWV VIP AISALIV ALM YR...	TMD (1)	23	27.1	1.18
		TMD (2)	17	28.9	1.67
Syb2	... K MMIL GV ICAIILIIIVYF ST	TMD (1)	22	54.9	2.50
		TMD (2)	20	53.1	2.66
PTP1B	... KPFL VNMCVATVLTAGAY LCYR...	TMD (1)	20	29.1	1.46
		TMD (2)	14	18.7	1.34
b5-scrambled-ops-28	... DSNSS WWASAI IATM IPLLVNVW YR...	TMD (1)	23	27.1	1.18
		TMD (2)	18	30.1	1.71
b5-HH-ops-28 ^b	... DSNS <u>SWWT</u> <u>AWV</u> <u>IP</u> <u>L</u> <u>L</u> <u>L</u> <u>V</u> <u>V</u> <u>ALM</u> YR...	TMD (1)	23	37.9	1.65
		TMD (2)	19	42.2	2.22
b5-Syb2-ops-28	... DSNSSW WTNMM IL GVICAIILIIIV YFYR...	TMD (1)	29	49.6	1.71
		TMD (2)	21	48.4	2.3
b5-Syb2mut-ops-28 ^b	... DSNSSW WTNMM G <u>IL</u> <u>GV</u> <u>CAG</u> <u>IL</u> —V YFYR...	TMD (1)	25	30.9	1.24
		TMD (2)	17	29.7	1.75

^aTMDs were defined either as the uncharged sequence close to the C terminus (sequence in between the charged residues, which are shown in bold; TMD (1)) or as the stretch with negative hydrophilicity according to the GES hydrophobicity scale (Engelman et al., 1986), computed with a window of seven residues (TMD (2)); indicated in italics. The TMD of b5-Syb2-ops-28 differs from the one of native Syb-2 (reported for comparison) in the flanking sequences derived from b5.

^bThe underlined characters in b5-HH-ops-28 and b5-Syb2mut-ops-28 indicate the residues that have been changed from the TMD of b5 and Syb2, respectively. In addition, the TMD of b5-HH-ops-28, which was derived from the rat protein, differs from the rabbit sequence (in b5-ops-28) by an I→V replacement at position 122 of the native polypeptide. This substitution has a negligible effect on the hydrophobicity of the TMD and no effect on translocation efficiency (unpublished data).

^cTotal hydrophobicity/number of residues in TMD.

physical/chemical characteristics of the two TMDs. As reported in Table I, the degree of the hydrophobicity of b5's TMD is considerably lower than that of Syb2's. To ascertain whether this difference in the hydrophobicity of the TMDs could account for the different translocation requirements of the two model proteins, we generated a construct with a TMD based on the one of b5 but modified by the substitution of four residues with more hydrophobic ones (b5-HH-ops-28; Fig. 1 e and Table I). As shown in Fig. 5 A, when tested in its ability to posttranslationally translocate across RMs or liposomes, this construct showed the same behavior as the b5-Syb2-ops-28 chimera, inserting efficiently into RMs but not into protein-free vesicles.

To confirm that TMD hydrophobicity and not a specific sequence is the principal parameter determining competence for unassisted insertion, we produced two further constructs: b5-scrambled-ops-28, in which the order of residues in b5's TMD was changed, and b5-Syb2mut-ops-28, in which the TMD of Syb2 was mutated to become less hydrophobic (Table I). As shown in Fig. 5 B, both of these constructs were able to insert into protein-free liposomes (lanes 7 and 12), albeit with somewhat reduced efficiency compared with the parent construct b5-ops-28 (lane 3).

Previous studies have reported that Syb2 depends on a microsomal trypsin-sensitive component for its insertion (Kutay et al., 1995; Kim et al., 1997; Abell et al., 2004). We asked whether Syb2's TMD is sufficient to confer this trypsin sensitivity and, if so, whether the increased hydrophobicity is the basis for this effect. RMs were treated with increasing concentrations of trypsin at 4°C to digest exposed proteins; efficacy of the treatment was verified by immunoblotting with antibodies against two ER integral membrane proteins, Sec61β and ribophorin I. As shown in Fig. 5 B, Sec61β was severely depleted already by treatment with 1 μg/ml trypsin, whereas ribophorin I was more resistant to the treatment and showed partial conversion to a shorter form lacking the cytosolic domain at higher trypsin concentration.

The trypsin-treated or mock-treated RMs were tested for transmembrane integration of the parent b5-ops-28 and of the two constructs with modified TMD (Fig. 5 C). As expected, trypsin treatment had no effect on b5-ops-28's ability to translocate its C terminus. In contrast, for the two constructs with more hydrophobic TMDs, even a mild trypsin digestion inactivated a microsomal component that is required for insertion of the constructs (Fig. 5 D, lanes 8–10 and 13–15).

PTP1B integrates into lipid bilayers by the same mechanism as b5

To investigate whether the ability to integrate into lipid bilayers without assistance from membrane proteins is a peculiarity of b5 or whether it is shared with other TA proteins, we analyzed the TMD sequence of 27 ER resident TA proteins (Table S3; available at <http://www.jcb.org/cgi/content/full/jcb.200608101/DC1>), searching for unassisted translocation candidates on the basis of low TMD hydrophobicity. For analysis, we selected PTP1B, whose TMD is similar in length and hydrophobicity to the one of b5 (Table I). PTP1B is a prototypic tyrosine phosphatase anchored to the ER membrane (Frangioni et al., 1992) and is involved in the regulation of numerous signaling events (Tonks, 2003). To adapt PTP1B to our assays, we appended the opsin epitope at its C terminus to obtain the PTP1B-ops-35 construct (Fig. 1 f and Table S1). As shown in Fig. 6 A, this PTP1B variant behaved exactly like b5-ops-28 in that (1) it posttranslationally inserted into RMs with the same efficiency as in cotranslational conditions, as judged by the appearance of a glycosylated form and by the recovery of a glycosylated PF, and (2) it inserted to the same extent into pure lipid liposomes, as shown by the presence of the nonglycosylated PF. Based on the ratio of glycosylated to nonglycosylated full-length protein, the efficiency of translocation of PTP1B-ops-35's C terminus was roughly the same as that of b5-ops-28 (Fig. 6 A, left).

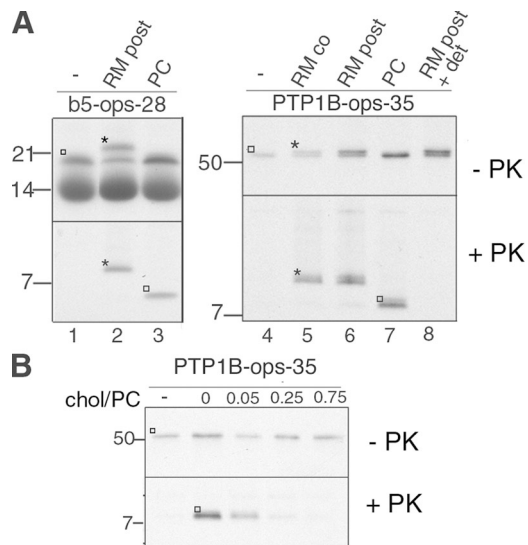


Figure 6. Unassisted insertion of the TA protein PTP1B. (A) PTP1B engineered with the opsin tag (Fig. 1 f) was tested for its ability to insert into RMs co- and posttranslationally or into PC liposomes (1.1 μg phospholipids/ μl in all cases). No PF was recovered in the absence of membranes or when detergent was present during PK digestion (right; lanes 4 and 8). The translocation behavior of b5-ops-28 was analyzed in parallel (left). (B) Effect of cholesterol on PTP1B translocation. Protein-free liposomes were prepared from PC-cholesterol mixtures as indicated and were tested for PTP1B-ops-35 insertion (1.3 μg phospholipids/ μl) as in A. Asterisks and boxes indicate the glycosylated and nonglycosylated products, respectively.

To further compare the mechanisms of the transmembrane integration of b5 and PTP1B, we analyzed the effect of the cholesterol content of liposomes on insertion efficiency (Fig. 6 B). Our previous work demonstrated that b5-ops-28 insertion is sharply inhibited by cholesterol levels only slightly higher than those normally found in the ER (Brambillasca et al., 2005). When in vitro-synthesized PTP1B-opsin-35 was incubated with liposomes containing increasing proportions of cholesterol, translocation was supported only by cholesterol-poor ones, as is the case for b5-ops-28 (Brambillasca et al., 2005). Thus, the unassisted insertion pathway described for b5 can also be exploited by other TA proteins whose TMDs have a similar degree of hydrophobicity.

Energy requirements for posttranslational transmembrane integration

Although the unassisted translocation process itself must be nucleotide independent, energy consumption by chaperones could be necessary to maintain the translocation substrates in a competent conformation. Because this possibility appeared especially likely in the case of the constructs with extended C-terminal domains, we compared the energy requirements for the integration of the longest construct capable of unassisted translocation (b5-ops-85) with those of the parent construct b5-ops-28. Nucleotides were depleted from translated samples by gel filtration, and samples were further diluted into a buffer compatible with b5 integration to reduce the final ATP concentration to 3 nM. As shown in Fig. 7 A, severe nucleotide depletion had no effect on the efficiency of the translocation of b5-ops-28

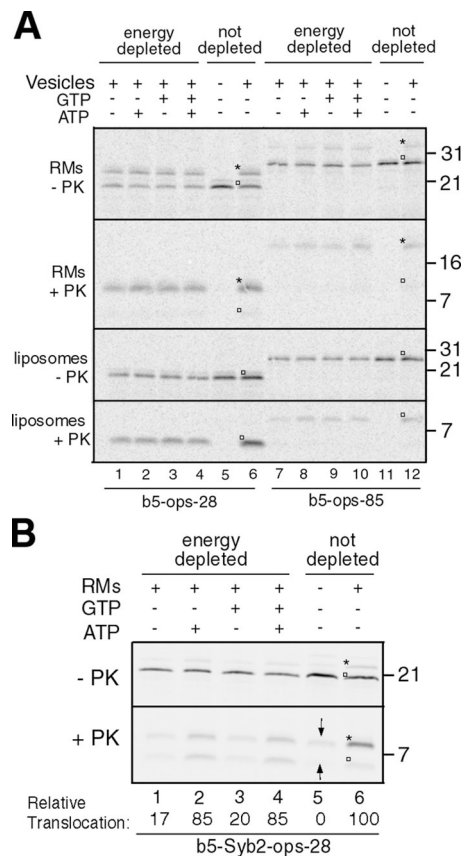


Figure 7. Energy requirements for the transmembrane integration of b5 constructs. (A) In vitro-synthesized b5-ops-28 and -85 were gel filtered and diluted in TB to obtain a final ATP concentration of 3 nM (lanes 1 and 7). The diluted samples were incubated with RMs or PC liposomes (two top and two bottom panels, respectively), both at 0.15 μg phospholipids/ μl , and were tested for insertion by protease protection. Where indicated, 0.5 mM ATP, 0.1 mM GTP, or both were added (lanes 2–4 and 8–10). Samples not subjected to nucleoside triphosphate depletion were analyzed in parallel. (B) In vitro-synthesized b5-Syb2-ops-28 was subjected to gel filtration and diluted in TB to reach a final ATP concentration of 5 nM during incubation with RMs (0.67 μg phospholipids/ μl). ATP, GTP, or both were added where indicated as described for A. Samples not subjected to gel filtration were analyzed in parallel, and background signals were generated after PK digestion in the absence of membranes, as indicated with arrows (lane 5). The percentage of translocation efficiency normalized to the efficiency obtained in mock-treated RMs and corrected for background and for the different input of gel-filtered and untreated samples is given below the lanes. Asterisks and boxes indicate the glycosylated and nonglycosylated products, respectively.

(compare lane 1 with 6) and, remarkably, had only a minor effect on that of the extended variant b5-ops-85 (compare lane 7 with 12) into either RMs (top panels) or liposomes (bottom panels). Furthermore, the addition of ATP, GTP, or both to restore nucleotide levels to those of the reticulocyte lysate was without effect on the translocation of b5-ops-28 (Fig. 7 A, lanes 2–4) and was mildly stimulatory on b5-ops-85 (Fig. 7 A, lanes 8–10). These results indicate that unassisted translocation not only of b5's C terminus but also of an extended polar domain appended to b5's TMD has extremely low energy requirements.

At variance with b5, Syb2's insertion is reported to be energy dependent (Kutay et al., 1995; Kim et al., 1997). We asked whether Syb2's TMD alone is responsible for this effect by

testing the transmembrane integration of the chimera b5-Syb2-ops-28 after ATP depletion. As shown in Fig. 7 B, after nucleotide depletion, insertion of the construct was severely reduced (lane 1). In agreement with Kutay et al. (1995) and Kim et al. (1997) but at variance with Abell et al. (2004), the readdition of ATP stimulated integration much more effectively than GTP. Thus, Syb2's TMD confers to the chimera all of the insertion requirements of Syb2 itself.

Discussion

Membrane proteins generally integrate into the lipid bilayer cotranslationally: during synthesis, the signal peptide or the first hydrophobic membrane-anchoring sequence to emerge from the ribosome is recognized by SRP, which delivers the nascent chain-ribosome complex to the translocation machinery of the ER membrane (Higy et al., 2004). The insertion pathway followed by TA proteins represents an exception to this rule because members of this class of proteins lack an N-terminal signal sequence, and their membrane-anchoring sequence is too close to the C terminus to become cotranslationally available to SRP (Borgese et al., 2003). Given the important functions of TA proteins in fundamental cellular processes, the molecular mechanisms underlying this posttranslational mode of transmembrane protein topogenesis are currently being studied in several laboratories.

The insertion pathway of a few TA proteins has been investigated in some detail, and important differences have been reported, as exemplified by Syb2 and b5. There is a general consensus that Syb2 requires a proteinaceous component of the ER membrane and energy to associate with RMs (Kutay et al., 1995; Kim et al., 1997, 1999; Abell et al., 2003, 2004), although there is disagreement regarding the nature of the involved protein and of the energy donor (ATP or GTP). In contrast, b5 integration occurs without assistance from any microsomal protein (Brambillasca et al., 2005) and appears to have very low energy requirements (Kim et al., 1997; Yabal et al., 2003).

In our previous work, we used a b5 variant carrying at its C terminus a 19-residue sequence derived from bovine opsin, which permitted us to monitor translocation by protection from proteolysis and/or glycosylation of the short translocated sequence (Brambillasca et al., 2005). The total length of the domain translocated without assistance, including the seven C-terminal residues of b5 itself, two linker residues, and the opsin sequence, was 28 residues. Two important questions were raised from this study: (1) what the maximum size is of the C-terminal polar domain that can be translocated without assistance and (2) what the basis is for the reported differences in the insertion requirements for different TA proteins. In the present study, we address both of these issues.

As for the first question, we find that unexpectedly long sequences (up to 85 residues) appended to b5's C terminus can be translocated across protein-free bilayers. Consistent with these *in vitro* observations, constructs with extended luminal domains could efficiently transfer their C terminus into the ER lumen of yeast mutants defective in translocon function. The process of unassisted integration appeared to be relatively

insensitive to the nature of the sequence to be translocated. The three classes of sequences that were attached to b5's C terminus and that translocated without assistance by membrane proteins in this study had different charge and different intracellular localizations when in their normal context: whereas the Hsp150 and opsin sequence are exoplasmic, the VSVG sequence was derived from the cytosolic tail of the viral glycoprotein. The VSVG tag differed from the other two also in its net positive charge, arguing against any role of an electrochemical gradient in the translocation process. Furthermore, the b5-VSVG-33 construct lacked the 19-residue opsin tag and the six C-terminal residues of b5, both of which are present in the other b5-based constructs, excluding a role for these residues in unassisted translocation.

Although unassisted transmembrane integration was relatively insensitive to the nature of sequences <100 residues appended to b5's C terminus and was unchanged by substitution of the cytosolic domain, it was dramatically affected by the properties of the TMD, pinpointing the basis of the differing requirements for transmembrane topogenesis of different TA proteins (the second question raised above). Substitution of b5's TMD with the more hydrophobic one of Syb2 conferred on the chimera all of the properties reported for native Syb2: incapacity to insert into liposomes, sharp inhibition of posttranslational integration by trypsin treatment of microsomes, and a requirement for energy (Kutay et al., 1995; Kim et al., 1997, 1999; Abell et al., 2004). These altered requirements for insertion were the result of the increased hydrophobicity of Syb2's TMD and not of a specific amino acid sequence because (1) an increase in the hydrophobicity of b5's TMD conferred by four amino acid substitutions was sufficient to completely block the unassisted translocation of even a short (28 residues) luminal domain, (2) mutations that decreased the hydrophobicity of Syb2's TMD conferred to the resulting construct the capability of unassisted insertion, and (3) scrambling the order of residues in b5's TMD generated a construct that was still capable of insertion into liposomes. In addition, we ruled out the possibility that the capacity for unassisted transmembrane integration is a peculiar feature of b5 by demonstrating the same capability of a nonrelated TA protein, PTP1B, which shares with b5 only the moderate hydrophobicity of its TMD.

Two other TA proteins whose membrane association has been investigated with binding assays, Nyv-1p (Steel et al., 2002) and Sec61 β (Abell et al., 2004), have requirements similar to those of Syb2, whereas another one, Bcl2, appears to be more like b5 (Kim et al., 1997). The behavior of these three TA proteins is predictable on the basis of the hydrophobicity of their TMDs (Table S3). Thus, TMD hydrophobicity appears to be a reliable predictor of whether or not assistance for transmembrane integration of a given TA protein is required.

Why should a more hydrophobic TMD preclude unassisted translocation? A possible explanation is that newly synthesized TA proteins with very hydrophobic TMDs require an ER protein and energy for delivery to the bilayer in a translocation-competent form rather than for the translocation step itself. It is known that the poor water solubility of hydrophobic peptides constitutes a major problem for their assembly into

performed lipid bilayers (Hunt et al., 1997; Reshetnyak et al., 2006). TA proteins with TMDs of limited hydrophobicity may be endowed with a certain degree of water solubility, which would allow them to directly access the bilayer. In contrast, TA proteins with very hydrophobic TMDs are probably rapidly sequestered into insoluble aggregates unless assisted by a chaperone, which might then require interaction with a cytosolically exposed ER protein to deliver the substrate to the bilayer. According to one study, this chaperone function is fulfilled by SRP and SRP receptor, acting in concert via a novel posttranslational mechanism (Abell et al., 2004). However, the observation supporting this conclusion is restricted to a small fraction of truncated Syb2 and Sec61 β nascent chains immediately after their release with puromycin. With naturally terminated Syb2 polypeptide incubated for longer times with RMs, a requirement for SRP receptor was not detected, and, in agreement with our data, ATP but not GTP was necessary for association of the polypeptide to RM membranes (Kutay et al., 1995). Thus, there appears to be more than one delivery pathway of TA proteins to the ER.

The finding reported here that a polar domain of nearly 100 residues placed downstream of an appropriate TMD can be translocated across protein-free bilayers was quite unexpected. How can such large polar domains make it across the hydrophobic core of the lipid bilayer? The free energy released upon insertion of the TMD (Moll and Thompson, 1994; Soekarjo et al., 1996) could possibly provide the driving force to overcome the kinetic energy barrier to translocation of the polar domain. Thus, the TMD of b5 and of other TA proteins endowed with a similar capacity for unassisted insertion would function as nanosyringes, catalyzing the translocation of their own C-terminal polar domain, provided that the lipid bilayer to be crossed is sufficiently disordered and that the length of the polar domain is not excessive. Indeed, increasing bilayer order by the addition of cholesterol (Straume and Litman, 1987) results in sharp inhibition of the process (Brambillasca et al., 2005; and this study); analogously, the constructs with long luminal domains, for which the kinetic barrier to translocation is presumably higher, integrate more slowly than those with shorter ones. Above a certain length of the C-terminal domain, the free energy of insertion of the TMD is presumably no longer sufficient to overcome the activation energy barrier opposing translocation.

An important question concerns the folding status of the C-terminal domain that is translocated without assistance. Structural experiments on the N-terminal domain of opsin have revealed that it easily folds to yield a compact structure and that this folding also occurs in the absence of *N*-glycosylation (Yeagle et al., 2000). In contrast, the 19-residue repeat domain of Hsp150 appended in one or more copies to the Hsp constructs is unstructured (Jämsä et al., 1995). Thus, we expected that these two sequences might have different requirements for translocation because of a different folding status. Instead, the very low energy requirements for the b5-ops-85 construct argue against an active unfolding process occurring before translocation. It is possible that the opsin domain was not folded under our experimental conditions or, alternatively, that it has sufficient conformational flexibility to spontaneously oscillate between folded

and unfolded states. The problem of the conformation of the luminal domain in the unassisted translocation pathway described here is the subject of ongoing investigation in our laboratory.

In addition to suggesting a mode of evolution of biomembranes, we believe that the results presented here have implications for contemporary membrane biogenesis and physiology. At present, we cannot rule out that TA proteins capable of transmembrane integration into protein-free liposomes *in vitro* are assisted *in vivo* by proteinaceous factors that accelerate their insertion, as found for M13 and Pf3 phage coat proteins (Samuelson et al., 2000). However, the equal efficiency of insertion into RMs and protein-free liposomes of PTP1B and of all constructs based on b5's TMDs as well as our previous demonstration of the absence in microsomal extracts of any protein stimulating b5-ops-28 translocation (Brambillasca et al., 2005) strongly suggest that the unassisted pathway characterized *in vitro* occurs also *in vivo*. Unassisted insertion may be relevant not only for TA proteins and for some bacterial proteins (van Dalen and de Kruijff, 2004) but also for thylakoid membrane biogenesis (Di Cola et al., 2005). Furthermore, given its potential overlap with the cotranslational pathway, it could represent a salvage mechanism for the insertion of some type II membrane proteins. It is well known that hydrophobicity is the principal parameter determining the affinity of signal sequences/anchors for SRP (Ng et al., 1996); thus, exactly those type II proteins that are more likely to escape from the cotranslational pathway would be better substrates for an unassisted salvage pathway. Finally, phenomena similar to the one reported here could underlie voltage- (Qiu et al., 1996) and lipid (Bogdanov et al., 2002)-dependent posttranslational rearrangements of membrane proteins that involve the translocation of large domains across the bilayer. Unconventional mechanisms of membrane biogenesis like the one reported here are thus likely to be of general significance and are likely to attract increasing attention in the coming years.

Materials and methods

Recombinant plasmids

The constructs used in this study are illustrated in Fig. 1. Sequences of the TMDs and of the C-terminal luminal domains are reported in Tables I and S1, respectively. All cDNAs for transcription/translation were cloned in the pGEM4 vector under the SP6 promoter and checked by sequencing. The recombinant plasmids were obtained by inserting cassettes of paired oligonucleotides or of PCR-amplified fragments into the parent plasmid b5-ops-28 (called b5-Nglyc in a previous study [Brambillasca et al., 2005]) or into its derived extended constructs. The cDNA coding for PTP1B, which was a gift from J. Chernoff (Fox Chase Cancer Center, Philadelphia, PA; Chernoff et al., 1990), was subcloned into pGEM4 and modified to contain the C-terminal opsin tag.

In vitro translocation assay

Transcription, translation in the reticulocyte lysate system (Promega), translocation reactions with RMs (a gift of R.S. Hegde, National Institute of Child Health and Human Development, Bethesda, MD) or liposomes prepared by extrusion, digestions with PK, immunoprecipitation with antiopsin monoclonal antibody (a gift of P. Hargrave, University of Florida, Gainesville, FL) used at 8 μ g/ml or anti-VSVG polyclonal antibody used at 7 μ g IgG/ml (Sigma-Aldrich), and SDS-PAGE analysis were performed as described previously (Brambillasca et al., 2005). In each experiment, samples contained equal amounts of phospholipids, as specified in the figure legends; phospholipid phosphorus in RMs was assayed according to Ames and Dubin (1960), whereas phospholipid concentration in liposome

suspensions was assessed by measuring the recovery of ^3H -PC (GE Healthcare) included in trace amounts in the lipid mixtures before ejection and extrusion. Incubations with vesicles were performed for 1 h unless specified otherwise. Dried gels were either exposed to film or to a phosphorimager screen (Storm; GE Healthcare). All quantifications were performed with the Storm phosphorimager using ImageQuant software (GE Healthcare). For comparisons of translocation efficiency based on the amount of PF generated, intensity values of the PF bands were normalized to the amount of the total full-length product generated in the corresponding translation reaction. In all figures, the numbers on the side of the panels indicate the position and molecular mass (in kilodaltons) of size markers.

ATP depletion of in vitro-translated samples

90 μl of each translation reaction was gel filtered on Sephadex-25 fine columns (0.8×4 cm; Bio-Rad Laboratories) equilibrated in a buffer suitable for translocation (translocation buffer [TB]; 50 mM Hepes, pH 7.2, 250 mM sorbitol, 70 mM KOAc, 5 mM K^+ EGTA, 2.5 mM $\text{Mg}(\text{OAc})_2$, and 2 mM DTT). 15 fractions of 90 μl were collected, and an aliquot of each was analyzed by SDS-PAGE to identify those with the maximum recovery of the radioactive protein. These were then assayed for ATP by the luciferin-luciferase procedure as previously described (Yabal et al., 2003) and used in the translocation assays after appropriate dilution in TB.

Trypsin treatment of microsomal membranes

Four equivalents of RMs (see Walter and Blobel, 1983 for a definition) were incubated for 1 h at 4°C with increasing concentrations of trypsin in a final volume of 50 μl trypsin buffer (50 mM triethanolamine-acetic acid, pH 7.5, 250 mM sucrose, and 1 mM DTT). Digestion was terminated by the addition of PMSF to 1 mM and aprotinin to 47 mTIU/ μl for 15 min at 4°C . Samples were then adjusted to 500 mM KOAc in a final volume of 200 μl , and RMs were sedimented at 63,000 rpm for 30 min in a rotor (TLA 100.3; Beckman Coulter), resuspended in 200 μl trypsin buffer, sedimented again, and finally resuspended at 0.5 equivalents/ μl in storage buffer (50 mM Hepes, pH 7.2, 250 mM sucrose, and 2 mM DTT). The effect of the trypsin treatment was assessed by Western blotting/ECL (Super-Signal West Pico; Pierce Chemical Co.) with antiribophorin I (antibody RIL3; a gift from G. Kreibich, New York University School of Medicine, New York, NY; Wiest et al., 1997) and anti-Sec61 β (provided by R.S. Hegde; Fons et al., 2003) antibodies.

Construction and metabolic labeling of yeast strains

The b5-ops constructs described in Fig. 1 (a and b) and in Tables I and S1 were subcloned between the *SUC2* promoter and the *ADH1* terminator in the *Escherichia coli* yeast shuttle vector pFL26. The resulting plasmids coding for b5-ops-28, -47, -85, and -125 were designated pKTH5013, pKTH5126, pKTH5181, and pKTH5237, respectively. The control yeast strain H1689 was created by integrating pKTH5013 into the *LEU* locus of strain H245 (see Table S2 for yeast strains). The sec61-3 strains were created by integrating the plasmids pKTH5103, pKTH5126, pKTH5181, and pKTH5237 into the *LEU* locus of the parental strain H257, generating strains H2270, H2237, H2240, and H2113, respectively. The sec61-2 strains were created by transforming the same plasmids into H1693, creating strains H1698, H2148, H2151, and H2116, respectively (Table S2).

The aforementioned described yeast cells were grown in synthetic complete medium in full (2%) glucose at the permissive temperature (24°C). Expression of the b5 constructs and parallel inactivation of Sec61p were obtained by up-regulating the *SUC* promoter in 2% raffinose (low glucose) medium at the restrictive temperature (38°C) for 1 h. Metabolic labeling of proteins with [^3S]methionine/cysteine (GE Healthcare), lysis, immunoprecipitation with antiopsin and anti-CPY antibodies, and endoglycosidase H digestion were performed as previously described (Yabal et al., 2003).

Online supplemental material

Fig. S1 shows the detergent controls for all of the b5 constructs and the lack of transmembrane integration of b5-hsp-104 and b5-ops-125 into liposomes. Fig. S2 shows the lack of effect of the substitution of b5's catalytic domain on unassisted translocation. Table S1 provides sequence information on the luminal domains of all of the constructs analyzed in this study. Table S2 is a list of the yeast strains used for the in vivo studies, and Table S3 is a list of 27 TA proteins analyzed for TMD hydrophobicity. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200608101/DC1>.

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