Organization of translocon complexes in ER membranes

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Abstract

Protein translocation in the ER (endoplasmic reticulum) and N-glycosylation are fundamental processes essential for the normal functioning of eukaryotic cells. They are the initial steps in the intracellular pathway that are followed by secretory proteins and membrane proteins of the endomembrane system and the plasma membrane. The translocation and concurrent N-glycosylation of these proteins take place on a large molecular machine, the TC (translocon complex), which is associated with membrane-bound polysomes. Segregation of TCs into a differentiated domain of the ER, the rough ER, may increase the efficiency of protein synthesis on membrane-bound polysomes. Our research is concerned with the assembly, functional organization and dynamics of the TCs in the ER, and their contribution to the functioning and the morphological appearance of this organelle. We hypothesize that the TCs form higher-order structures defining the rough domain of the ER. These structures, which are immobilized or diffuse slowly in the plain of the ER membrane, may be formed and stabilized by mRNAs interconnecting the TCs, by cytoskeletal elements and/or by hypothetical proteins that form links between the TCs. We have established the M3/18 cell line, which expresses the GFP (green fluorescent protein)–Dad1 fusion protein quantitatively and functionally incorporated into the OST (oligosaccharyltransferase). GFP–Dad1 can be used as a reporter molecule for the lateral mobility of the TCs since the OST is tightly associated with the complex. As determined by FRAP (fluorescence recovery after photobleaching), the lateral mobility of GFP–Dad1-tagged TCs was much more restricted than expected from the estimated size of the TC and can be affected by the functional state of the TCs. Currently, we are studying the possible involvement of cytoskeletal elements in the organization of the TCs. Our data suggest that microtubules also play a role in the immobilization of the TCs.

Components of the TC (translocon complex)

The RER [rough ER (endoplasmic reticulum)] is the site where proteins made on membrane-bound polysomes are inserted into or translocated across the ER membrane. Here, a complex molecular machinery effects the signal-sequence-mediated targeting, co-translational translocation and processing of nascent polypeptide chains [1]. Present evidence suggests that the proteins concerned with these functions form an oligomeric structure, the TC, which has Sec61p at its core [2,3] (Figure 1). Our research on the TC had focused primarily on the structure and function of the OST (oligosaccharyltransferase). This complex has been identified and purified from various species [4–6]. Ribophorin I and II were originally described in our laboratory as the first membrane proteins specific to the RER [7]. From previous findings concerning the near-neighbour relationship of the ribophorins with other components of the TC, it is very likely that the OST is intimately associated with membrane-bound ribosomes and Sec61p [8–10]. It had been shown that when rough microsomes are solubilized with certain non-ionic detergents, membrane-bound polysomes co-sediment with the TC, including the OST [7–9,11]. Also, the ribophorins can be chemically cross-linked to the 60 S ribosomal subunit [12], and affinity-purified polyclonal and anti-peptide antibodies against ribophorin I and II inhibit the translocation of nascent polypeptide chains [13].

Overall, the TC is composed of more than 25 polypeptides with a total of at least 60 TMDs (transmembrane domains), and it has a molecular mass of about 850 kDa [2,3]. The best evidence for the size and composition of the TC comes from recent structural studies on membrane-bound ribosomes isolated under conditions that preserve their interaction with the TC. The overall diameter of the TC is about 125 Å [10]. An active TC carries a ribosome (250 Å in diameter), which has a molecular mass of about 5000 kDa and may also be associated with cytosolic factors [2,14–16]. This would increase the mass to 7000 kDa, with an additional 500 kDa contributed by each 1 kb of mRNA. If active TCs organized into a polysome synthesizing a protein of 20 kDa, the structure will have the molecular mass of more than 36600 kDa and have at least 305 TMDs. The molecular mass and number of TMDs will increase proportionally with the size of the protein encoded by an mRNA. Such a large
Figure 1 | Stages of TC assembly

The individual subunits of Sec61p or the OST (A) are rather quickly integrated into oligomeric structures (B), which are assembled into the TC (C). The TCs are recruited into a polysome (D). Some of the membrane-bound polysomes may be segregated in differentiated domains of the RER (E). Since the diameter of a ribosome (∼250 Å) is significantly larger than that of the TC (125 Å) [10], RER-specific proteins (solid ellipses) associated with TCs could provide physical interaction between adjacent TCs to form large molecular arrays. Attachment of microtubules via ER membrane proteins such as CLIMP-63 [17] could also restrain the lateral mobility of the TC.

structure with TMDs interacting with the lipid bilayer, and domains exposed to the cytosol and the ER lumen, may be subjected to considerable friction that would impede its lateral diffusion.

FRAP (fluorescence recovery after photobleaching) as a tool for studying the functional organization of the TCs

The fluid mosaic membrane model that was proposed about 30 years ago emphasized the free mobility of membrane proteins in the plane of the membrane [18]. Since then it has been found that many membrane proteins are not completely free to diffuse in the plane of the lipid bilayer. Such proteins may be retarded in their lateral diffusion or completely immobilized through interactions with cytoskeletal elements, with extracellular matrix proteins or by becoming a part of large oligomeric arrays. These insights were gained at least in part through biophysical methods including FRAP [19,20]. In FRAP analysis, a small area of a cell that contains a fluorescent probe, such as GFP (green fluorescent protein), is exposed to irreversible photobleaching radiation by an intense laser flash. The fluorescence recovery of the bleached area, through exchange of bleached for non-bleached molecules, is recorded with an attenuated laser beam. The rate of recovery is used to calculate an effective diffusion constant \( D_{\text{eff}} \) for the mobile fraction of the GFP-tagged structure [20]. The lateral diffusion of integral membrane proteins in the lipid bilayer decreases with the natural logarithm of the radius of the diffusing molecule [21]:

\[
D_{\text{eff}} = k(\ln c/a - 0.577)
\]

where \( D_{\text{eff}} \) is the diffusion constant, \( k \) and \( c \) are constants that take into account the membrane bilayer thickness, viscosity and the viscosity of the surrounding medium, and \( a \) is the radius of the TMDs of the diffusing membrane protein complex. Edidin and co-workers [22] used this equation to calculate the size of the GFP-tagged H2L4 or TAP1 complex.

We have recently described M3/18 cells expressing a GFP–Dad1 fusion protein that is functionally integrated into the OST as one of the subunits of the complex [23]. We have demonstrated that the mobility of GFP–Dad1-tagged active TCs is about seven times lower than that of the highly mobile LBR (lamin B receptor)–GFP construct [23]. These results provided the first direct evidence that the lateral mobility of TCs in ER membranes of the living cells is severely impaired. Using eqn (1) and assuming that friction is only caused by interactions of TMDs with the lipid bilayer, we calculated that the TCs would be organized into large rafts occupying an area of approx. \( 5.7 \times 10^2 \) µm². Considering the state and morphology of the RER in these cells, one has to assume that, besides the physical size of the rafts, other mechanisms are expected to play a role in restricting the mobility of TCs.

Mechanisms that affect the lateral mobility of TCs

The restricted mobility of the TCs was suggested by previous findings. (i) In hepatocytes, which have a well-differentiated RER and smooth ER, a sharp transition between these two continuous membrane domains is observed. (ii) The morphology of the RER (flattened cisternae) and the smooth ER (contorted tubules) is strikingly different. (iii) In grazing sections of the RER, membrane-bound polysomes are arranged in regular figures that would not be expected if TCs carrying them were freely mobile. (iv) Large aggregates of polysomes containing components of the TC can be isolated from rough microsomes solubilized with non-ionic detergents.
The lateral mobility of TCs can be affected by mechanisms involving cytoskeletal elements, such as microtubules, or proteins that bridge adjacent TCs. Biochemical evidence, as well as our FRAP results, suggested that membrane-bound polysomes and thus TCs may be segregated in a RER-specific domain. By direct or indirect interactions among TCs they may form large rafts, which may provide a more efficient environment for protein synthesis on membrane-bound polysomes. In pancreatic AR42J cells, we have shown that the induction of amylase synthesis and secretion by dimethasone results in a striking rearrangement of the RER from a tubulo-vesicular configuration into stacks of cisternae without an increase in the number of membrane-bound ribosomes. Morphological research dating back to the early work by Palade described particles on the surface of ER membranes that are arranged in characteristic patterns, such as circles, spirals, loops or hairpins, which have been later recognized as membrane-bound polysomes. Christensen's laboratory has shown that large mRNAs encoding thyroglobulin (330 kDa) or collagen (150 kDa) generate ribosome arrangements in the form of spirals or double rows that contain more than 30 ribosomes. It was found that the spacing between the centres of ribosomes along the mRNA was quite constant (about 250 Å) and independent of the type of the translated mRNA. The regular spacing between ribosomes requires that during translation the rate of initiation and elongation remains constant. Irregularities in these processes, including ‘ribosome pausing’ during translation, would result in prominent gaps and clustering of ribosomes, unless the TCs are part of a rigid, raft-like structure. Furthermore, the average actual length of the mRNA segments that interconnect adjacent ribosomes is about double the length than would be needed to interconnect adjacent ribosomes. Therefore, the distance between ribosomes, which is constant, is not defined by the length of the interconnecting mRNA segment or the diameter of the ribosome. The authors proposed a model in which ‘...adjacent strands of mRNA in large bound polysomes would be maintained at a relatively constant distance from one another by spacing mechanisms, perhaps involving membrane proteins of the RER’. The diameter of the TC is about 125 Å. Since the distance between the ribosomes is much larger than the diameter of the TC, linker proteins would be required if TCs form raft-like arrays (Figures 1 and 2B).

The lateral mobility of TCs is affected by depolymerization of microtubules

Another possible explanation for the low lateral mobility of GFP-Dad in M3/18 cells is that the active TCs are partially immobilized by interaction of membrane-bound ribosomes with cytosolic components including cytoskeletal elements. It has been shown that the injected dextran particles of the size of ribosomes are restrained in their diffusion but still mobile. The high protein concentration in the cytoplasm does not, however, explain the immobilization of ribosome-size particles by itself, since the particles are still mobile in protein solutions with a protein concentration comparable with that found in the cytoplasm. The slow diffusion of TCs observed in our previous FRAP experiments may therefore be caused, at least in part, by interaction of membrane-bound ribosomes with the cytoskeletal elements. The ER is known to form an elaborate network of tubules and cisternae that are thought to be stabilized by microtubules. Recent ultrastructural reconstructions of the three-dimensional arrangement of cellular organelles showed in fact that microtubules are in very close proximity to the ER. Moreover, it has been shown that the RER-specific membrane protein CLIMP-63, which itself can form oligomers, apparently tethers microtubules to the ER. There are two possibilities with regard to how cytoskeletal elements may interact with TCs. (a) They are forming a grid-like structure parallel to the plane of the ER membrane. Even if they are not directly attached to ER membranes, they may provide fence-like barriers for the diffusion of membrane-bound ribosomes. (b) Cytoskeletal elements are attached to the ER membrane remnants with ribosomes attached to a proteinaceous fibrillar meshwork that was insensitive to RNase treatment.
membranes via receptors or adaptors, thus forming a post-like arrangement perpendicular to the ER membrane. In this arrangement, cytoskeletal elements may affect predominantly polysomal assemblies that get entangled via their mRNAs. Both possible arrangements of microtubules with regard to the plane of the ER membrane are illustrated in Figure 2(A).

To test whether cytoskeletal elements interfere with the lateral mobility of TCs we have performed FRAP experiments on M3/18 cells that were treated with drugs that affect the state of the cytoskeleton (Figure 3). The experiments were performed as described previously [23], except that they were done on a heated stage (39.5 °C) and not at room temperature. Under these conditions the lateral mobility of TCs (Figure 3, bars 4 and 5 respectively). Treatment of the cells with lantrunculin B, a drug that stabilizes microtubules, resulted in a faster diffusion (Figure 3, bar 3). Treatment of the cells with puromycin, which causes termination of protein synthesis, resulted in a 3-fold increase in the diffusion rate of the TCs (Figure 3, bar 2). In contrast, treatment of the cells with taxol, which causes depolymerization of microtubules, resulted in a faster diffusion of the TCs. Since we did not detect any inhibition of protein synthesis in vinblastin-treated cells, the observed increases in the lateral mobility of the TCs strongly suggest that microtubules in close proximity to the ER may affect the mobility of membrane-bound polysomes.

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References

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Figure 3 | Treatment of the cells with vinblastin results in increased lateral mobility of the TCs

M3/18 cells were grown at 39.5 °C and left untreated (1) or treated with (2) cycloheximide (80 µM; 30 min), (3) puromycin (100 µM; 30 min), (4) lantrunculin B (10 µM; 2 h), (5) taxol (5 µM; 4 h) or (6) vinblastin (10 µM; 2 h). FRAP experiments and data analyses were carried out as described previously [23] except that a heated stage (39.5 °C) was used.

[Insert Figure 3 here]
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