The role of translocon peptide channel in the transport of small molecules across the endoplasmic reticulum membrane

Doctoral Theses

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Budapest
2007

Introduction

Several enzyme activities localized in the luminal compartment of the ER require the transmembrane fluxes of their substrates, products and/or cofactors. Accordingly, variety of structurally unrelated compounds is (or should be) able to cross the ER membrane, but only a few transporters have been reported so far. In comparison with other organelles transporters of the ER are less known. In the background of this phenomenon might be the technical difficulty of transport measurements and of the purification and reconstruction of the transporters. The ER is a eukaryotic organelle which has no obvious relationship with any bacterial ancestor so the *in silico* approach for transporter gene identification is less fruitful. Xenogenous molecules can be transported into the lumen also and the existence of specific transporters for them can be excluded.

The conspicuous discrepancy between the functionally characterized transport processes and the transport proteins identified at molecular level suggest that non-specific transporters can participate in the substrate supply of intraluminal enzymes. Many of the ER transport processes are based on antiport mechanism, a non specialized channel can ensure the counter molecule influx for these processes.

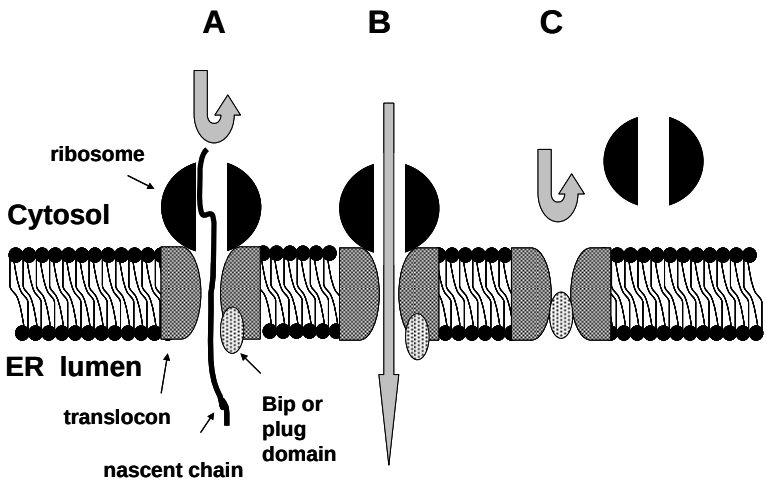
Several observations proved that the presence of translocons has significant influence on the permeability of the ER membrane.

Cotranslational protein translocation and integration into the rough ER membrane occur at sites termed translocon. The aqueous lumen and the large diameter of this channel

permit the permeation of small molecules. During translocation the lumen of the channel is occupied by the translocating peptide (A). When the ribosome is released from the translocon it results a closed state also by the luminal Bip or the plug domain (C). At the end of translation most of the ribosomes do not dissociate from the channel, therefore a free pore remains transitionally in the membrane of the ER (B).

The role of translocon peptide channel was proved in the permeation of a small neutral molecule, the 4MαG. The non-specific Ca^{2+}-leakage can be mediated also by the translocon.

The aim of our work was to prove the translocon role in the non-specific transport of small anions, which can ensure the substrate supply of intraluminal enzymes with high latency.



Aims

- Demonstration of the role of the translocon peptide channel in the non specific permeability of rat liver microsomes by the measurement of sucrose and citrate fluxes.
- Examination of the participation of the translocon in the substrate transport of intraluminal enzymes (UDP-glucuronosyltransferase, glucose-6-phosphatase, β-glucuronidase) by direct transport measurements and by the detection of intraluminal enzyme activities.
- If the peptide channel contributes to the transport of small molecules, by modifying its opening we can affect the permeability also. Therefore we were interested in how the transport changes by the releasing of ribosomes or by closing the channel by Sec61 antibody.
- Comparison of the permeability of RER and SER by the measurement of the latencies of intraluminal enzymes.

Methods

Preparation of rat liver microsomes

Liver microsomes were prepared from fed male Wistar rats (180-230 g body weight) by differential centrifugation, then immediately frozen in liquid nitrogen and kept in liquid nitrogen until use. The protein content of the microsomes was determined by BioRad Protein assay. Integrity of microsomal vesicles was assessed by

measuring the latency of intraluminal enzymes (mannose-6-phosphatase, UDP-glucuronosyltransferase).

Preparation of rough and smooth subfractions

Rough and smooth sub-fractions of liver microsomes were separated using sucrose gradient containing CsCl. The purity of the subfractions was evaluated by immunoblotting of Sec61α.

Transport measurements by light scattering techniques

The permeability of the microsomal membranes to sucrose and citrate was also measured by continuous detection of the osmotically-induced changes in size and shape of microsomal vesicles. Addition of a small volume (< 5% of the total incubation volume) of a concentrated osmolyte solution results in a rapid increase in light scattering (due to the shrinkage of the vesicles). This peak is followed by a gradual decrease in light scattering (which reflects vesicle swelling due to progressive equilibration of the osmolyte concentration between the extra- and intravesicular spaces). The relative height of the peak and the slope of the curve depend on the permeability of the vesicular membrane.

Rapid filtration

For the measurement of the uptake and the accumulation of radiolabeled compounds, liver microsomes were incubated with the substrates plus their radiolabeled analogues. At the indicated time points, samples were

rapidly filtered through cellulose acetate/nitrate filter membrane. The radioactivity associated with microsomes retained by filters was measured by liquid scintillation counting. In each experiment, alamethicin was added to parallel incubates to distinguish the intravesicular and the bound radioactivity.

Determination of latencies

In the case of enzymes with intraluminal active centers the rate of their substrate transport can affect the enzyme activity measured on microsomes. For the characterization of such enzymes latency is used, which means the difference between the measured and total enzyme activity in the percent of the total activity. For the determination of latency the total enzyme activity is needed which reflects the state when the substrates can freely reach the active site of the enzyme without the limiting effect of the transport. To determine this all enzyme activity assay were performed using the pore forming alamethicin also.

Determination of intraluminal enzyme activities

Microsomal UDP-glucuronosyltransferase activity was measured by using *p*-nitrophenol and UDP-glucuronic acid as substrates. The activity was calculated on the basis of decreasing p-nitrophenol absorbance. Microsomal β-glucuronidase activity was measured photometrically with *p*-nitrophenyl glucuronide and phenolphthalein glucuronide as substrates. Glucose-6-phosphatase activity was measured by determining the

amount of phosphate released by the enzymes with the malachite green reagent.

Western blot

The Sec61 content of the different microsomal subfractions was determined by immunoblot analysis. Equal amount of microsomal proteins were separated by SDS-PAGE and transferred to PVDF filter membranes by electroblotting. The equal loading was verified by Ponceau Red dying. The primary antibody labeled a band of the expected molecular weight (38 kDa) which was evaluated by densitometry.

Modifying the permeability of the translocon

The translocon peptide channel can be opened with puromycin, a tRNA-analogue antibiotic, which releases the nascent chain from the lumen of the channel. Anisomycin is a known antagonist of puromycin, which is only effective if added beforehand. Pretreatment with EDTA or with high salt buffer detach ribosomes from the ER surface resulting in a closed state of the peptide channel. An antibody binding to the Sec61 protein of the translocon occludes the pore of the channel.

Statistical analysis

Statistical comparisons were made by ANOVA followed by a Dunnett's test or by a Tukey's test or by paired two-tailed Student's *t* test.

Results

Puromycin increases the permeability of rat liver microsomes to citrate and sucrose.

In the first set of experiments, the effect of puromycin was validated by using membrane non-permeant compounds. Sucrose and citrate are not metabolized inside the ER; therefore, presumably they do not have specific membrane transporters. The permeation of these compounds was checked by the light scattering method. Addition of sucrose or citrate to microsomal suspensions resulted in a rapid increase in the light scattering signal reflecting a shrinkage of vesicles, which was followed by a very slow decrease in the signal reflecting a the swelling phase due to the influx. The slope of the second phase was steeper in microsomes preincubated with puromycin, indicating that puromycin increased the permeability of the vesicles towards both sucrose and citrate. Upon alamethicin addition the signal returned to the basal level, indicating that the osmotic effects did not alter the vesicular structure of the microsomes.

The effect of puromycin on microsomal sucrose uptake

To control whether this increase in permeability can really be attributed to the translocon pore (and not to a non-specific effect on the membrane), the effect of puromycin was evaluated either in total (non-fractionated) and smooth microsomes, in which translocons are underrepresented. The uptake was measured with rapid filtration technique, by using

radiolabeled sucrose. The basal rate of sucrose uptake was higher in total as compared to smooth microsomes, calculated either on protein or on phospholipid basis. Puromycin caused an increase in the rate of sucrose uptake of total microsomes only.

The effect of puromycin on microsomal anion uptake

The uptake of various anions by rat liver microsomes was detected by using radiolabeled compounds by rapid filtration. The control time course of UDP-glucuronate influx showed the characteristic initial overshoot followed by a decline, as it has been reported by others. A completely different curve was gained in the presence of puromycin; both the overshoot and the declination phases disappeared and an equilibrating uptake was detected. Puromycin was ineffective in case of glucose-6-phosphate and glutathione transport, presumably due to their specific, high capacity transporter.

Puromycin promotes substrate access to intraluminal enzymes of the ER

UDP-glucuronosyltransferases, glucose-6-phosphatase and β-glucuronidase are ER enzymes with active sites located in the lumen. Hence, the ER membrane represents a barrier for their substrates; consequently, the activity of these enzymes is reduced in native microsomal vesicles. Agents that disrupt the integrity or increase the permeability of the membrane cause an increase in the activity of these enzymes.

Microsomal UDP-glucuronosyltransferase activity was measured by using *p*-nitrophenol and UDP-glucuronic acid as substrates. The activity in native microsomal vesicles was less than 10% of the total activity measured in permeabilized vesicles. Addition of puromycin resulted in a concentration-dependent increase in the activity; 30% activation was observed at 0.5 mM puromycin concentration. However, puromycin was ineffective in alamethicin-permeabilized microsomes, which indicates that the effect was likely due to the improved access of UDP-glucuronic acid the enzyme to the enzyme and not a direct activation of the enzyme itself.

Microsomal β-glucuronidase activity was measured with *p*-nitrophenyl glucuronide and phenolphthalein glucuronide as substrates. In accordance with previous results, the enzyme had a moderate latency in control vesicles. Puromycin did not increase β-glucuronidase activity.

The catalytic subunit of glucose-6-phosphatase is able to hydrolyze hexose phosphates other than glucose-6-phosphate. The specificity of glucose-6-phosphatase activity is guaranteed by an ER transport protein, which strictly specific to glucose-6-phosphate. Consequently, the latency of the enzyme is lower with glucose-6-phosphate as substrate than with other hexose phosphates, *e.g.* with mannose-6-phosphate. Therefore, changes in hexose-6-phosphatase activities upon puromycin addition were monitored by using both substrates. Addition of puromycin resulted in a concentration-dependent increase in mannose-6-phosphatase activity. A maximal activation of about 15

% was observed. Puromycin was ineffective in alamethicin-permeabilized microsomes, which argues against a direct activation of the enzyme. Puromycin was unable to increase the activity when glucose-6-phosphate was used as substrate.

The effect of puromycin on UDP-glucuronosyltransferase activity can be prevented by a translocon-binding antibody, by the puromycin antagonist anisomycin and by the release of ribosomes

It has been reported that an antibody (or its proteolytic fragment) binding to the Sec61 protein of the translocon occludes the pore of the channel. Therefore, the translocon-specific effect of puromycin was controlled by investigating the effect of this antibody on UDP-glucuronosyltransferase activity. Specific binding of the antibody was checked by immunoblotting the microsomal proteins. As expected, the addition of the antibody to microsomes counteracted the effect of puromycin. Moreover, it decreased the activity of UDP-glucuronosyltransferase even in the absence of puromycin . The inhibition was specific, since neither the heat-denatured antibody, nor an anti-rabbit secondary antibody was effective.
Anisomycin is a known antagonist of puromycin, which is only effective if added beforehand. Anisomycin has been successfully used in microsomal experiments to prevent various translocon-dependent effects of puromycin. In our experiments, puromycin failed to increase UDP-glucuronosyltransferase activity when

added after anisomycin. If anisomycin was added after puromycin, it was ineffective.
The release of ribosomes from the surface of microsomal vesicles also prevented the effect of puromycin. Pretreatment with EDTA, which is known to detach ribosomes, decreased UDP-glucuronosyltransferase activity by 37%, and completely abolished the effect of puromycin. Puromycin was also ineffective in microsomes pretreated with a high-salt buffer, another maneuver that also removes ribosomes from the microsomal membrane. Treatment of native microsomes with high-salt buffer did not affect UDP-glucuronosyltransferase activity; in permeabilized microsomes the buffer slightly increased the activity.

Different membrane permeability in smooth and rough microsomes

Since recent observations indicate that non-translating empty ribosomes are present on the surface of the ER, it can be supposed that this condition contributes to the permeability of the ER membrane. By using the above approach, we compared the latency of UDP-glucuronosyltransferase, glucose- and mannose-6-phosphatase activities in rough (*i.e.* translocon-rich) and smooth microsomal fractions. All these enzyme activities were present in both microsomal fractions. The latency of UDP-glucuronosyltransferase activity was much greater in smooth vesicles. The latency of glucose-6-phosphatase enzyme measured with glucose-6-phosphate was similar in the two fractions. However, when mannose-6-phosphate was added as substrate, a higher latency was

found in smooth microsomes. The purity of the subfractions was evaluated by immunoblotting of Sec61α. Comparing to the total microsomal fraction, 1.5-fold enrichment in the rough and fivefold impoverishment in the smooth subfraction was observed by the densitometry of the corresponding bands.

Conclusions

- Translocon protein channel can mediate the permeation of small molecular weight anions (UDP-glucuronic acid, mannose-6-phosphate) across the ER membrane.
- The phenomenon may play a role in the substrate supply and/or product elimination of some intraluminal enzymatic activities in the ER.
- The translocon peptide channel plays important role in the non-specific permeability of the ER membrane.

Publications related to the dissertation

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Publication not related to the dissertation

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