Protein insertion into the endoplasmic reticulum of permeabilized cells

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Abstract **We have established efficient translocation of newly synthesized proteins into the endoplasmic reticulum of permeabilized Mel Juso cells. By site-specific photo-crosslinking we show that translocating polypeptide chains contact the same components of permeabilized cells ER as in dog pancreas rough microsomes. This cellular assay system has the potential to overcome the limitations of isolated microsomes in investigating the molecular environment of a newly synthesized protein after they have left the ER translocation site.**

Key words: Cell permeabilization; Site-specific photo-crosslinking; Endoplasmic reticulum; Protein translocation

1. Introduction

Protein translocation across the membrane of the endoplasmic reticulum (ER) has been studied very successfully by using dog pancreas rough microsomes in a cell-free translation system (for review see [1]). The signal recognition particle (SRP) was shown to interact with signal sequences of nascent polypeptides and arrests further elongation until the ER membrane has been contacted and the nascent polypeptide chain enters the protein-conducting channel. In combination with several crosslinking strategies, constituents of the ER protein-conducting channel, the Sec61 complex, the TRAM protein and phospholipids were identified [2–5]. As rough microsomes are vesicles, only the first steps of the secretory pathway can be studied.

To investigate the transport of a protein from the ER translocation site to other cellular compartments, e.g. the Golgi, the plasma membrane or endosomes, insertion into the ER of an entire cell has to be accomplished. Different strategies have been used to introduce exogenous molecules into perforated cells. The plasma membrane has been mechanically ruptured [6], bacterial pore forming toxins have been used [7] or detergents that preferentially permeabilize the plasma membrane have been applied [8]. Among the detergents are saponin and digitonin that insert into membranes containing cholesterol and perforate them [9]. We have used cells permeabilized with saponin and show that a membrane protein synthesized in the presence of these cells is efficiently inserted into the ER.

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2. Materials and methods

2.1. Preparation of semi-intact cells

Mel Juso cells [10] were grown to confluency in 75 cm^2 plates, washed twice in PBS and released from the dish by incubation in PBS, 0.8 mM EDTA. All subsequent steps were carried out at room temperature. Cells were washed in 3 ml buffer H (165 mM KOAc, 2 mM $Mg(OAc)_2$, 50 mM HEPES-KOH pH 7.4), and sedimented for 30 s at 5000 rpm in an Heräus Biofuge 15. The cells were resuspended in 150 μl buffer H containing 0.01% saponin (Sigma). After 10 min incubation 600 μl buffer H were added, the cells were pelleted as above and resuspended in 60 μl buffer H for immediate use in a translation assay.

2.2. Plasmids and transcription

Plasmids encoding human invariant chain Ii (pGEM3Ii) and IiTAG41 (pGEM3IiTAG41) as well as preprolactin PPLTAG18 (pGEM4PPLTAG18) were described previously [2,3,5]. The cDNA coding for the ER retained Ii33 was made in pET3 vector (Novagen) by mutation of pETIi coding for Ii. Two oligonucleotide cassettes were ligated between the *Nde*I and *Xba*I sites to account for the 16 amino acid N-terminal extension of Ii33. The same procedure was applied to prepare pETIi33TAG2. Messenger RNA was synthesized in vitro from linearized plasmid using T7 RNA polymerase.

2.3. Translations and site-specific photo-crosslinking

Translation in the wheat germ cell-free system, amber suppressor tRNA mediated incorporation of (Tmd)Phe and photo-crosslinking were performed essentially as described [2], except that permeabilized cells (2 μl per 25 μl translation mixture) were used instead of dog pancreas rough microsomes. Proteins in the translation and translocation assays were either precipitated by the addition of 4 vol. of 10% TCA, 25% aceton or analyzed by immunoprecipitation with anti-TRAM or anti-Sec61α antibodies [2]. Where indicated, samples were treated with bee venom phospholipase A_2 (Boehringer Mannheim) as described previously [2]. Samples were analyzed by SDS-PAGE using 12%–18% gradient gels according to Laemmli [11] or 14% T, 3% C gels according to Schägger and von Jagow [12] (for Fig. 4B). Polypeptides containing [^{35}S]methionine were detected by either autoradiography using Kodak XAR-5 film or by imaging using a Fujix BAS1000 phosphoImager (for Fig. 4A and B).

2.4. Measurements of marker enzymes

Lactate dehydrogenase activity was measured as described by Wroblewski et al. [13]. Alkaline α-glucosidase was assessed by measuring the increase of fluorescence (Ex 358 nm/Em 452 nm) upon incubation with methylumbelliferyl glucopyranoside at pH 9.2 [14].

2.5. ER visualization in DiOC stained cells

The ER of control cells and of cells permeabilized with saponin were fluorochromed by the vital stain $DiOC_6(3)$ (3,3′-dihexyloxacarbocyanine-iodide; 2 mg/ml; Eastman Kodak) at room temperature [15], viewed on a confocal laser scanning microscope (Leica), and the observed images documented using Agfa 100 film.

3. Results

3.1. Saponin permeabilization of Mel Juso cells

Treatment of cells with saponin has been described as a method for permeabilization of their membranes [9]. Saponin preferentially permeabilizes membranes with high cholesterol

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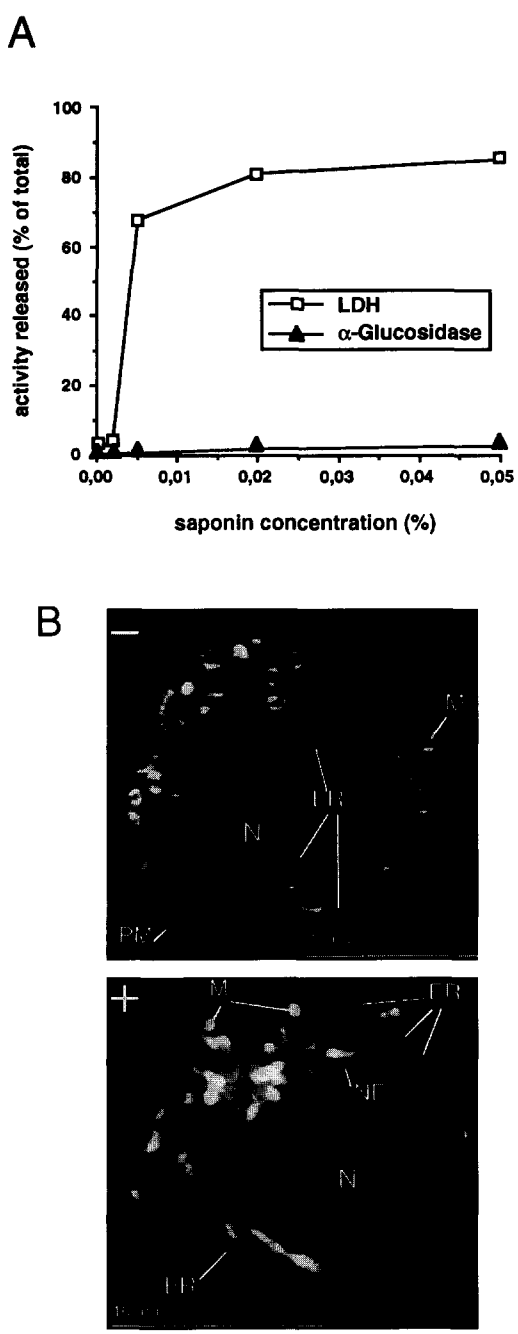

Fig. 1. Effect of saponin on the ER of Mel Juso cells. (A) Release of LDH and α-glucosidase upon saponin treatment. Cells were treated with different saponin concentrations and subsequently sedimented by centrifugation. LDH and α-glucosidase activities were measured both in supernatants and pellets. (B) Structural integrity of the ER in Mel Juso cells (−, upper panel) and cells permeabilized with 0,01% saponin (+, lower panel). Vital staining of the ER with the fluorochrome $DiOC_6(3)$. Stained cells were investigated with a confocal laser scanning microscope. ER: endoplasmic reticulum, M: mitochondria, PM: plasma membrane, N: nucleus, NE: nuclear envelope.

content like the plasma membrane. As the ER membrane is low in cholesterol, it is resistant to saponin even at concentrations up to 0.5% [9]. To assess permeabilization of Mel Juso cells with saponin, we measured the release of lactate dehydrogenase (LDH), a marker enzyme for soluble cytosolic proteins, upon incubation of cells with increasing concentrations of saponin (Fig. 1A). With concentrations of ≥0.01% saponin, most of the LDH activity was released from the cells. Alkaline α-glucosidase, an ER marker enzyme, was not released even at a saponin concentration of 0.05% (Fig. 1A). The effect of saponin on the integrity of the ER was further investigated on a confocal laser scanning microscope. Mel Juso cells were treated with 0.01% saponin and ER and mitochondria [15] were stained with the fluorochrome $DiOC_6(3)$. As shown in Fig. 1B, a clear perinuclear reticular staining pattern, characteristic for the ER network, can be observed both in control cells and in saponin treated cells.

3.2. Translation and translocation in the presence of permeabilized cells

To show whether a membrane protein can be inserted into the ER membrane, we translated the type II membrane protein invariant chain (Fig. 2A) in a wheat germ cell-free system in the presence of permeabilized Mel Juso cells. Without the addition of exogenous mRNA, no translation was observed (Fig. 2B, lane 3). When Ii33 mRNA was added to the translation reaction, selection of the two initiation codons (Fig. 2A) results in the synthesis of two proteins, Ii31 and Ii33 (Fig. 2B, lane 1). Both translation products were inserted into the ER which is indicated by a shift to a higher molecular weight consistent with glycosylation at two sites (Fig. 2B, lane 1 and Fig. 3A, lanes 1 and 4). Translation and translocation were as efficient with permeabilized cells as with microsomes (Fig. 2B, lane 2).

Correct membrane insertion of Ii was examined by treatment with proteinase K. A significant proportion of membrane inserted Ii was protected from the protease (Fig. 2C, lane 2). The increased electrophoretic mobility upon proteolysis was due to digestion of the N-terminal cytoplasmic tail of Ii. This result indicates that Ii inserted into the ER membrane in the same orientation as in vivo (Fig. 2C, lane 2) [16]. When membranes were solubilized with Triton X-100 and treated with proteinase K, Ii was completely digested (Fig. 2C, lane 3).

Interactions taking place between a newly synthesized protein and components of the ER translocation apparatus were investigated by site-specific photo-crosslinking. A chemically amino acylated amber suppressor tRNA was used to mediate the incorporation of the photoactivatable amino acid L-4′-(trifluoromethyl)3*H*-diazirin-3-yl]phenylalanine [(Tme)Phe] at a selected position (in response to a UAG stop codon) within the coding region of a mRNA [2,5]. First, suppression efficiency was asessed by translating Ii33TAG2 mRNA which contains an UAG codon at position 2. Without the addition of suppressor tRNA, no translation from the first ATG initiation codon (see Fig. 2A) was observed and exclusively Ii31 was synthesized (Fig. 3A, lane 2). In the presence of membranes, Ii31 became glycosylated (Ii31*; Fig 3A, lane 5). When suppressor tRNA was present during translation, also Ii33 and glycosylated Ii33 (Ii33*) were synthesized (Fig. 3A, lane 3 and 6). Translation, suppression and glycosylation were as efficient with permeabilized cells as with rough microsomes (Fig. 3A vs. B).

For crosslinking experiments we used IiTAG41 to introduce (Tmd)Phe at position 41 within the hydrophobic core region of the Ii signal anchor sequence. IiTAG41 was previously used to demonstrate contact of translocating Ii with lipids and proteinaceous components of the protein-conducting channel in dog pancreas rough microsomes [2]. In permeabilized Mel Juso

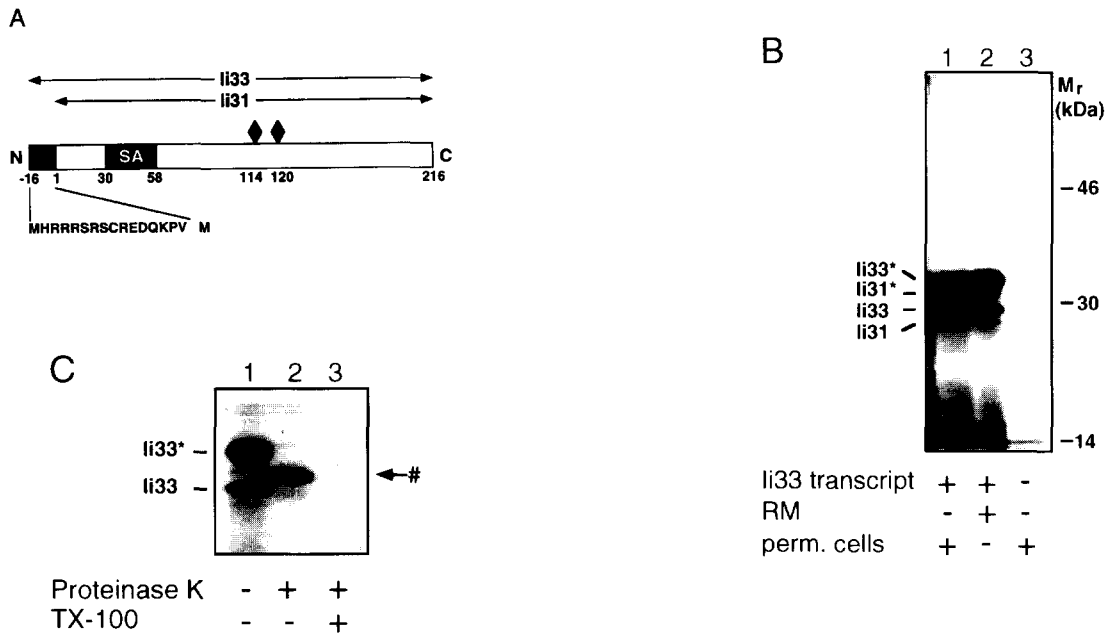

Fig. 2. Protein translocation into the ER of saponin-treated Mel Juso cells. (A) Outline of Ii31 and Ii33. The 16 amino acid extension of Ii33 is shown by a grey box and the amino acid sequence. The black box indicates the hydrophobic core region of the signal anchor (SA) sequence; potential *N*-glycosylation sites are indicated by diamonds. (B) Translation of Ii33 mRNA in the presence of permeabilized cells (lane 1) or dog pancreas rough microsomes (lane 2). Background translation (no Ii33 mRNA) is shown in lane 3. Ii31* and Ii33* indicate glycosylated translation products. (C) Protease protection of translocated Ii33. Glycosylated Ii33* is partially protected from proteinase K (#, lane 2), only the cytoplasmically exposed N-terminus (~3 kDa) is accessible to the protease. After solubilization of membranes with Triton X-100, Ii33* is completely digested (lane 3).

cells, treatment of IiTAG41/103 translocation intermediates after crosslinking with bee venom phospholipase A_2 or immunoprecipitaion with anti TRAM antibodies revealed contact of the nascent polypeptide chain to phospholipids and to the TRAM protein (Fig. 4A).

A similar experiment was performed with a 86 amino acid long translocation intermediate of the secretory protein preprolactin, with (Tmd)Phe incorporated within the hydrophobic core region of the signal sequence (PPLTAG18/86). In dog pancreas rough microsomes, PPLTAG18/86 was previously crosslinked to Sec61α [2,5]. As shown by immunoprecipitaion, PPLTAG18/86 was also crosslinked to Sec61α in permeabilized Mel Juso cells (Fig. 4B).

4. Discussion

In the present study, we show that it is possible to open the plasma membrane while keeping ER structures intact enough to observe efficient membrane insertion and translocation of newly synthesized proteins into the ER. Efficient targeting of newly synthesized proteins to the ER translocation site was confirmed by site-specific photo-crosslinking which revealed

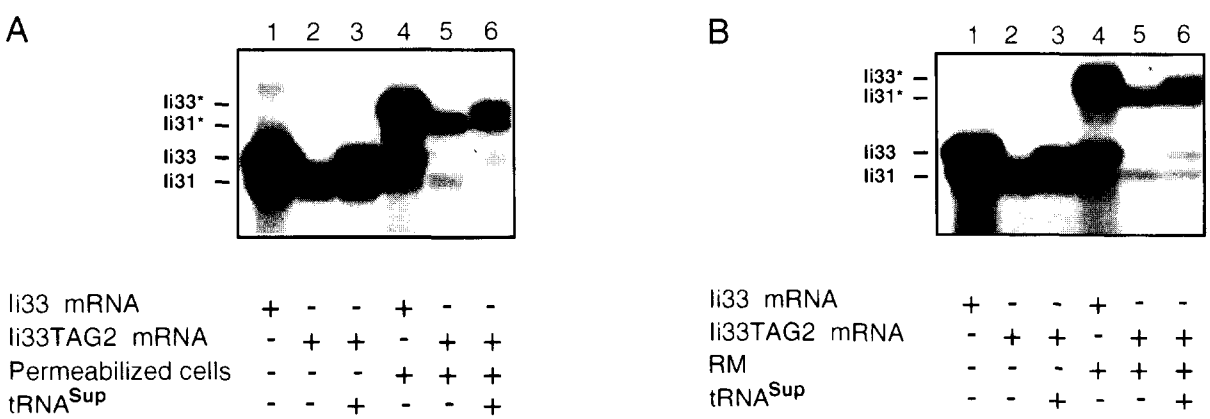

Fig. 3. Suppresion efficiency in the presence of saponin-treated Mel Juso cells. (A) Ii33 mRNA (control, lanes 1 and 4) and Ii33TAG2 mRNA (lanes 2, 3, 5 and 6) were translated in the absence (lanes 1, 2 and 3) or in the presence (lanes 4, 5 and 6) of saponin-treated Mel Juso cells. Without suppressor tRNA (lane 2 and 5), Ii33TAG2 mRNA is not translated. Only the second methionine is used to initiate translation of Ii31, which becomes glycosylated (Ii31*) in the presence of membranes (lane 5). When suppressor tRNA is added (lanes 3 and 6), Ii33 is synthesized and becomes glycosylated (Ii33*) in the presence of membranes (lane 6). (B) Translations of Ii33 mRNA and Ii33TAG2 mRNA as in (A) but in the presence of dog pancreas rough microsomes instead of permeabilized Mel Juso cells.

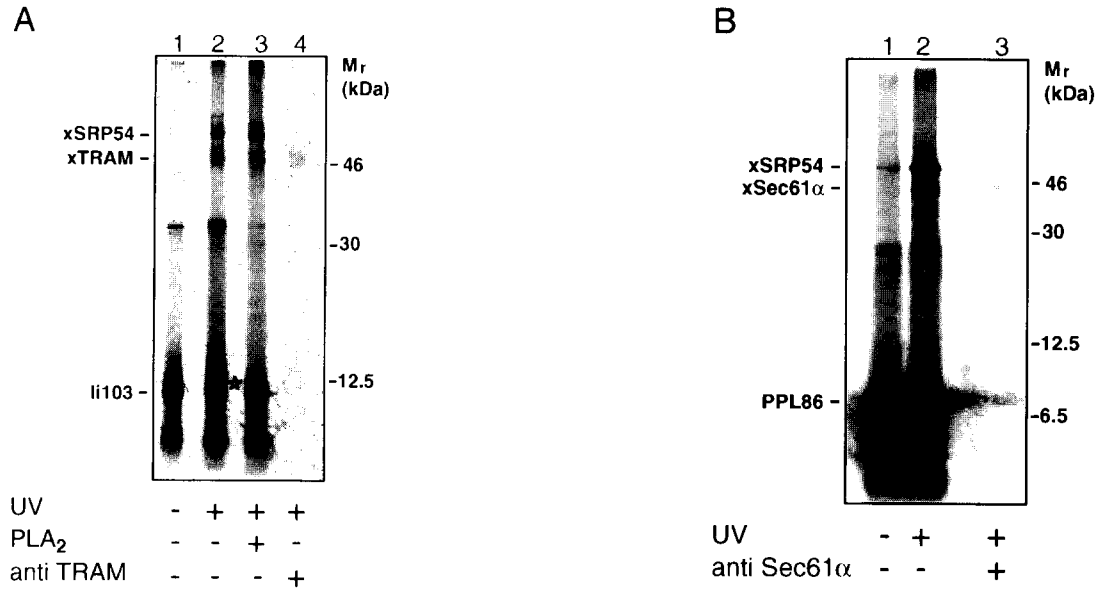

Fig. 4. Photo-crosslinking with IiTAG41/103 and PPLTAG18/86 to membrane components. Radiolabeled IiTAG41/103 (A) and PPLTAG18/86 (B) translocation intermediates were synthesized in the presence of SRP and saponin-treated Mel Juso cells. Samples were UV irradiated and analyzed for membrane-associated crosslink products either directly (lanes 1 and 2) or upon treatment with phospholipase A_2 (A, lane 3) or immunoprecipitation with anti-TRAM (A, lane 4), and anti-Sec61α (B, lane 3) antibodies. Crosslinks to lipids are indicated by a star (A, lane 2).

contact of translocating polypeptides to the human counterparts of the protein conducting channel, TRAM protein, Sec61α and phospholipid. Furthermore, correct membrane insertion of a type II membrane protein (Ii) was demonstrated by processing (glycosylation) and by protease protection of the C-terminal domain. Thus, saponin permeabilized cells are an easy to use in vitro system to investigate insertion of proteins into the ER membrane in the context of a cell.

To apply the site-specific photo-crosslinking approach, permeabilized cells have to be supplemented with exogenous mRNA and suppressor tRNA charged with the crosslinking reagent. In addition, components for efficient translation have to be re-added as the cells have been washed after saponin treatment. As expected when wheat germ extract was used, translation was efficient. In addition, membrane insertion and translocation into the ER were efficient, comparable to results with microsomes. This indicates that the cells were permeabilized such that large particles as ribosome-nascent chain complexes could 'enter' the cell. At low concentrations, saponin is believed to destabilize the plasma membrane so that it can no longer withstand the colloid osmotic pressure and holes ranging in size from 100 nm to 1 μm are formed [9,17]. Semi-intact cells prepared by hypotonic swelling have previously been shown to be active in transport from the ER to the Golgi complex [18]. We therefore believe that although the ER of saponin permeabilized cells is partially dilated due to osmotic swelling [17], the combination of cell permeabilization and site-specific photo-crosslinking provides a promising tool to investigate the fate of a newly synthesized protein once it has left the ER translocation site.

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