J. Plant Biol. 37(3) : 293~299 (1994)

Translocation of Seed Storage Proteins into Microsomes Isolated from Rice Endosperm Cells

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Developing rice endosperm cells display two morphologically distinct rough endoplasmic reticulum (ER) membranes, the cisternae ER (C-ER) and the protein body ER (PB-ER), the latter delimiting the prolamine protein bodies. We (Li *et al.*, 1993) have recently shown that the storage protein mRNAs are not randomly distributed on these ER types; the C-ER is enriched for glutelin mRNAs, whereas the PB-ER harbors predominantly prolamine transcripts. To address whether these ER types have different capacities to translate these mRNAs and translocate their proteins into the lumen, a microsomal fraction enriched in C-ER vesicles was prepared from developing rice seeds. When present in an *in vitro* translation system, the microsomes were able to proteolytically remove the signal peptide and internalize both preproglutelin and preprolamine within the microsomal vesicles. Of the two species, preprolamine was more effectively translocated and processed. These results suggest that the C-ER has the capacity to recognize and bind both storage protein mRNAs during protein synthesis. Moreover, efficient translocation and processing of glutelin requires additional factors that are deficient or absent in the *in vitro* system.

Keywords : *Oryza sativa* L., storage protein, glutelin, prolamine, transport

Proteins that are sorted to their final cellular destination through the secretory pathway are translated on the endoplasmic reticulum (ER) in eucaryotic cells. The translation of these proteins, however, is initiated in the cytosol. Upon emergence of the signal peptide during translation the nascent polypeptide chain-ribosome-mRNA complex is targeted to the ER (Walter and Lingappa, 1986). Biochemically purified microsome fractions enriched for rough ER vesicles, *i.e.* ER membranes with attached ribosomes, possess all the factors essential to direct the targeting of the translational complex to the membrane, and subsequent proteolytic processing of the signal peptide and translocation of the protein across the membrane (Walter and Lingappa, 1986). Among the major factors identified by *in vitro* reconstitution experiments are the signal recognition particle (SRP) that mediates the targeting of the translation complex onto the ER membrane, the ER-receptor as well as other membrane associated ancillary factors that mediate polypeptide translocation, and the signal peptidase that cleaves the signal peptide (Walter and Lingappa, 1986).

Plant cells appear to employ a similar mechanism to sort vacuolar-localized and secretory proteins. Isolated microsomes from wheat germ (Prehn *et al.*, 1987) and maize endosperm (Campos *et al.*, 1989a) contain the necessary factors required for targeting the translational complex to the membrane, and translocation and processing of the protein. SRP-like factors have been identified from plant cells and are functionally identical to their mammalian counterparts (Campos *et al.*, 1989a; Prehn *et al.*, 1987). Differences, however, may exist between the plant and mammlian SRPs as viewed by the heterogeneity of the 7S RNA component. At least three species of 7S RNAs are present in a population of plant SRPs

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(Campos *et al.*, 1989b; Marshallsay *et al.*, 1989), whereas only a single RNA species has been identified in mammalian cells (Walter and Lingappa, 1986). The functional significance, if any, of the diversity of plant SRPs in protein translocation remains unclear.

Rice endosperm provides an excellent system to analyze intracellular protein transport. This tissue accumulates both types of storage proteins that are ubiquitously present in most plant seeds. The major storage proteins of rice endosperm are the insoluble glutelins, proteins homologous to the salt-soluble 11S globulins of legumes. In addition, rice also accumulates the alcohol-soluble prolamines, the storage protein typically observed in cereals, but at reduced levels as compared to the glutelins. The cellular pathways of transport and packaging of these storage proteins are distinct. Glutelins are targeted to a vacuolar compartment *via* the Golgi complex, whereas prolamines are retained within the ER lumen. The ER membrane delimiting the prolamine protein body (protein body ER or PB-ER) is often associated with ribosomes, suggesting that prolamines may be synthesized specifically on these membranes (Krishnan *et al.*, 1986; Yamagata and Tanaka, 1986). Indeed, we have recently demonstrated that the PB-ER membranes harbor predominantly prolamine mRNA as compared to glutelin transcripts (Li *et al.*, 1993). In contrast, the cisternae ER (C-ER) membranes are enriched in glutelin transcripts despite the somewhat higher steady-state levels of prolamine mRNAs during most stages of seed development (Kim *et al.*, 1993). The segregation of glutelin and prolamine mRNAs on the C-ER and the PB-ER, respectively, raises an interesting question as to whether the C-ER membrane may be more efficient in binding the translational complex, and translocating and processing glutelin as compared to prolamine. As an initial step to elucidate the biochemical basis of the segregation of these mRNAs on these ER membranes, we isolated an enriched C-ER fraction and specifically addressed whether this membrane fraction possessed varying capacities to translocate and process these storage proteins in an *in vitro* protein synthesizing system. Here, we show that the enriched C-ER vesicles are capable of translocating and processing both rice prolamine and glutelin with prolamine being more efficiently translocated and processed.

MATERIALS AND METHODS

Plant material

Rice (M-201) was grown in an environment controlled growth chamber under conditions described by Krishnan *et al.* (1986). Developing rice seeds (14-15 d after flowering) were harvested, immediately frozen in liquid nitrogen and stored at −80℃.

Preparation of microsomes

Twenty grams of seeds were pulverized with a coffee mill and suspended in 40 mL of buffer A [20 mM Tris-HCl, pH 8.5, 50 mM $MgCl_2$, 100 mM KCl, 1 mM dithiothreitol (DTT) and 0.2 M sucrose] similar to that described by Larkins and Hurkman (1978). The homogenate was filtered through two layers of nylon cloth (80 mesh) and then centrifuged at 5000 *g* for 10 min. The supernatant was collected and centrifuged at 30,000 *g* for 30 min. The supernatant fluid was carefully removed and the pellet was suspended in 5 mL of buffer A, supplemented with $CaCl_2$ to 2 mM, and treated with 150 units of micrococcal nuclease (Boehringer Mannhem) for 10 min at room temperature. The nuclease digestion was terminated by the addition of 4 mM ethylene glycol-bis(β-aminoethyl ether (EGTA). After incubation on ice for 10 min, the sample was diluted to 10 mL with buffer A, layered onto a 35% (w/v) sucrose cushion in homogenization buffer and then centrifuged at 100,000 *g* for 1 h at 4℃. The supernatant was thoroughly removed and the pellet was processed either for electron microscopic (EM) examination or for protein translocation assay.

For EM examination the pellet was fixed overnight at 4℃ in 10 mM Hepes, pH 7.2, 5 mM $CaCl_2$, 1.5% (v/v) glutaraldehyde and 2% paraformaldehyde. After extensive washing with cacodylate buffer (10 mM, pH 7.2), the sample was postfixed in 1% (w/v) OsO_4 in cacodylate buffer for 1 h. The sample was washed with cacodylate buffer, dehydrated with ethanol, embedded in L. R. White resin, sectioned and examined with an electron microscope as described previously (Kim *et al.*, 1988; Li and Franceschi, 1989). For protein translocation assay, the pellet was suspended in 1 mL of storage buffer (5 mM Hepes, pH 7.6, 1 mM Mg acetate, 1 mM DTT and 20%

glycerol). The resulting suspension was clarified of large membranous aggregates by spinning in a microcentrifuge for 5 min. After adjusting the concentration to 100 A_{260}/mL the microsome sample was divided into small aliquots, frozen in liquid nitrogen and stored at −80℃.

In vitro transcription

In vitro transcriptions of pProl 17 (Kim and Okita, 1988) and pGlut 22 (Okita *et al.*, 1989) were conducted in the presence of the GpppG, the cap analog, according to the manufacturer's recommendation (Promega) using T3 or T7 RNA polymerase. After transcription the reaction mix was treated with RNase free DNase to remove the DNA template. The synthetic glutelin and prolamine mRNAs were then precipitated with sodium acetate and ethanol (Maniatis *et al.*, 1982) and their concentrations estimated by absorbance at A_{260}.

Protein translocation asssay

Protein translocation assay was carried out in a wheat germ cell-free translation system using synthetic mRNAs transcribed from pGlut 22 (Okita *et al.*, 1989) for glutelin or pProl 17 (Kim and Okita, 1988) for prolamine. The wheat germ translation system was essentially the same as described by Anderson *et al.* (1983) with 2.5 mM Mg^{2+} and 65 mM K^{+}. The mRNAs were translated in a 25 μL reaction mix in the presence or absence of microsomes (4.0 mL). Protein translocation activity was detected by the appearance of the mature size polypeptides generated by proteolytic removal of the signal peptides and protease protection assay. Protease protection assays were performed according to Connolly and Gilmore (1986). Briefly, after translation the reaction mix was incubated on ice for 10 min and then diluted to 50 μL with an equal volume of 2X buffer B (100 mM Tricine-acetate, pH 8.0, 300 mM K^{+} acetate, 5.0 mM Mg^{2+} acetate and 4.0 mM $CaCl_2$) or Buffer C (100 mM Tricine-acetate, pH 8.0, 300 mM K^{+} acetate, 5.0 mM Mg^{2+} acetate and 2% Triton X-100). Twenty-five mg of proteinase K (Sigma) was added to the mixture which was then incubated on ice for 30 min. The protease digestion was terminated by the addition of 5 μL of 100 mM phenylmethylsulbonyl (PMSF). After incubation on ice for 10 min, an equal volume of 2X SDS sample buffer (Connolly and Gilmore, 1986) was added to the mixture and immediately boiled for 5 min. The resulting samples were resolved on either a 10% (for glutelin) or 15% (for prolamine) SDS polyacrylamide gel. The gels were treated with Resolution (EM Corp), dried onto filter papers and exposed to X-ray film.

Miscellaneous

A protein body fraction was prepared from the 5000*g* pellet during microsome preparation (see above). The pellet was suspended in 25 mL homogenization buffer and the suspension was centrifuged at 500*g* for 5 min to remove starch grains and nuclei. The supernatant was collected and centrifuged at 2000*g* for 10 min to pellet the protein body fraction. The protein concentrations of the microsome and protein body fractions were determined according to Bradford (1976). Twenty-five μg of protein samples of microsomes and protein body fractions were resolved on 15% SDS polyacrylamide gels. The polypeptide bands were visualized by silver staining (Ohsawa and Ebata, 1983) or transferred to nitrocellulose membrane and immunoblotted with a mixture of antibodies against the glutelin large subunit and prolamine (Krishnan and Okita, 1986). The bound antibodies were probed with ^{125}I-Protein A as described (Krishnan and Okita, 1986).

RESULTS AND DISCUSSION

Electron microscopic examination of the enriched rough ER membrane preparation revealed that it contained some smooth membranes but was comprised primarily of small vesicles with attached ribosomes, presumably derived from the C-ER and thus referred to as microsomes (Fig. 1). As viewed by SDS-PAGE, the protein profile of the microsome fraction exhibited numerous polypeptides (Fig. 2A). The protein body fraction (see Materials and Methods for preparation), however, exhibited different protein profile to that of microsome fraction (Fig. 2A). The major polypeptide bands displayed by the protein body fraction were storage proteins. Both the glutelin large and small subunits displayed a negative staining pattern when visualized with reduced silver, while the prolamine polypeptide of about 14 kD in size was positively stained (Fig. 2A). However,

Fig. 1. Electron micrograph of a microsome fraction enriched for cisternae ER (C-ER) membranes. An enriched C-ER fraction was obtained from developing rice cells by differential centrifugation as discussed in the Materials and Methods section. Bar represents 0.35 μm.

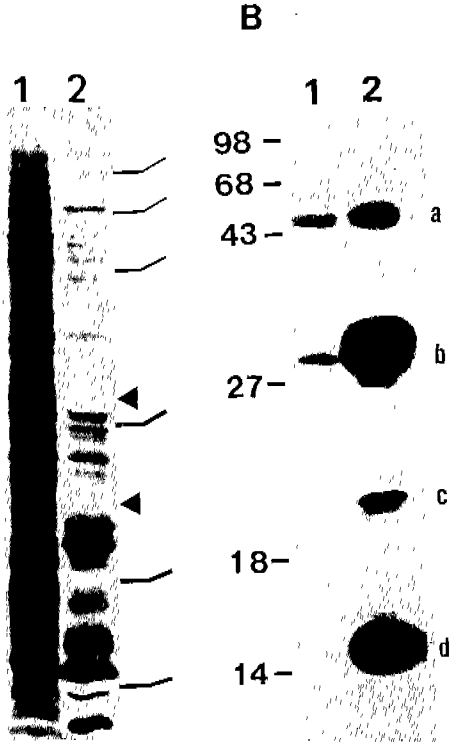

Fig. 2. Polypeptide composition of microsome fractions and enriched protein body. The microsome fraction enriched for C-ER (lanes 1) and protein body fraction (lanes 2) were resolved by SDS polyacrylamide gel electrophoresis and silver stained (panel A) or subjected to immunoblot analysis using a mixture of anti-prolamine and anti-glutelin large subunit (panel B). The arrowheads denote the location of the glutelin large and small subunits. The size in kD and location of the protein molecular weight standards are indicated between panels A and B. a, proglutelin; b, glutelin large subunit; c, glutelin small subunit; d, prolamine polypeptide.

the presence of common polypeptide bands were also observed in the microsome and protein body fractions, probably reflecting the presence of ribosomes in these two fractions (Krishnan *et al.*, 1986; Yamagata and Tanaka, 1986; Li *et al.*, 1993) as well as the fact that the protein body fraction was significantly contaminated with large C-ER membrane complexes (Li *et al.*, 1993).

To determine the extent of contamination of the microsome fraction by small protein bodies, protein samples of the microsome and protein body fractions were resolved on a SDS polyacrylamide gel, transferred to nitrocellulose membrane and subjected to immunoblot analysis using a mixture of antibodies raised against prolamine and glutelin large subunit (Krishnan and Okita, 1986). The latter antibody preparation is able to recognize not only the glutelin large subunit but also the post-translationally uncleaved proglutelin and, to a much lesser extent, the glutelin small subunit. As expected, significant amounts of prolamines and glutelins, particularly the two glutelin subnunits, were present in the protein body fraction (Fig. 2B, lane 2). In contrast, only small amounts of glutelins (Fig. 2B, lane 1, bands b and c) and prolamines (band d) were found in the microsome fraction. The proglutelin polypeptides (band a) were present at a higher proportion, as compared to the glutelin large subunit (band b), in the microsome fraction than in protein body fraction (Fig. 2B, lane 1). This observation is consistent with the temporary residence of these proteins in the rough ER lumen, which are subsequently transported to the protein body *via* the Golgi complex (Krishnan *et al.*, 1986). Since proglutelins are presumably cleaved into the large and small subunits during their transport from the Golgi or within the protein bodies, the presence of a small amount of glutelin subunits in the microsome fraction (Fig. 2B) suggested that this fraction was slightly contaminated with either Golgi/transport vesicles or small protein bodies. The low level of prolamines in the microsome fraction (Fig. 2B) is consistent with the fact that prolamines are retained and accumulated in the rough ER lumen.

To test for their ability to transport and process the seed storage proteins, prolamines and glutelins, the microsomes were first treated with micrococcal nuclease to digest mRNAs associated with these membranes. The microsomes were then added to an

in vitro translation mix in the presence of synthetic glutelin or prolamine mRNAs and the protein synthesis reaction was carried out at 30°C for 50 min at optimal Mg^{2+} and K^+ concentrations, conditions optimal for the translation of these mRNAs (results not shown). Fig. 3 depicts the results of a fluorogram of the polypeptide products resolved by SDS-PAGE produced by the *in vitro* translation of synthetic glutelin and prolamine transcripts in the presence or absence of microsomal membranes. In the absence of microsomal membranes, the 53 kD preproglutelin and 16 kD preprolamine polypeptides were synthesized, a result initally observed by Krishnan and Okita (1986). When the *in vitro* translation reaction was carried out in the presence of microsomes, however, additional smaller mol wt species of 51 kD and 14 kD corresponding to the proglutelin and mature prolamine polypeptides, respectively, were evident. The differences in molecular sizes of the preproteins and the corresponding processed smaller mol wt counterparts are consistent with the predicted sizes of their signal peptides as determined by DNA sequence analysis of full length cDNA clones of these transcripts (Kim and Okita, 1988; Okita *et al.*, 1989). To determine whether the processed polypeptides were internalized within microsomal vesicles, the reaction was post-incubated with proteinase K. As shown in Fig. 3, only the processed smaller mol wt polypeptides and not the larger preproteins were resistant to proteinase K digestion. When the proteinase K protection experiments was conducted in the presence of detergent, which disrupts the integrity of the microsomal membranes (Fig. 3), these processed protein bands were no longer detectable, indicating that the protection of the processed proteins against proteinase K digestion was dependent on membrane integrity. The results of the protease protection assay support the view that the smaller polypeptides (proglutelin and mature prolamine) were localized within the microsomes, whereas the preproglutelin and preprolamine (larger polypeptides) were excluded from these membranes and susceptible to proteolysis. The translocation and processing of glutelin and prolamine occurred co-translationally, as addition of microsomes to the mix after translation did not result in the cleavage of the signal peptides or protection of the synthesized products (data not shown). Increasing amounts of microsomes in the *in vitro* translation reaction up to 16 A_{260} units/mL resulted in increasing translocation and processing activities, but addition of higher amounts of microsomal membranes resulted in severe inhibition of translation (data not shown). Similar observations have been made in other protein translation/translocation systems (Walter *et al.*, 1981; Campos *et al.*, 1989a).

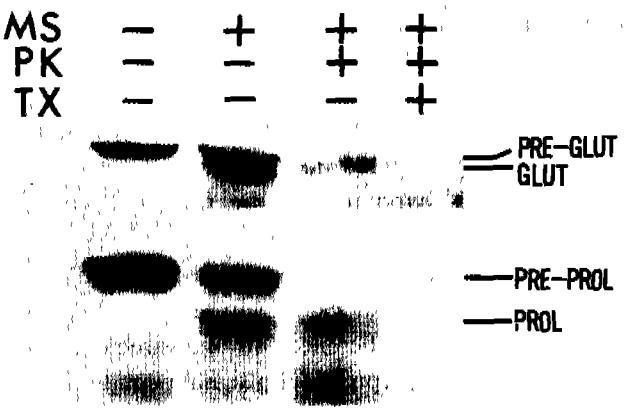

Fig. 3. *In vitro* translation of synthetic glutelin and prolamine mRNAs and their translocation and processing by rice microsomal membranes. Translation assays were conducted in the absence or presence of microsomal membranes (MS). Some reactions were then subsequently treated with proteinase K (PK) in the presence or absence of Triton X-100 (TX).

As observed with the maize endosperm microsomes (Campos *et al.*, 1989a), the translocation and processing activities in this system was relatively inefficient. About 30-40% of the prolamines were translocated and processed and even a smaller proportion was evident for glutelin (Fig. 3). The inefficient translocation and processing activities may stem from the heterologous system where one or more factors, *e.g.* SRP, may be limited. It has been shown that supplementation of SRP to a heterogeneous system can improve the efficiency of translocation and processing to 100% (Walter *et al.*, 1981).

In a previous study, we have shown that the C-ER were enriched (about 2-fold) with glutelin transcripts as compared to prolamine mRNAs (Li *et al.*, 1993). The uptake and processing of prolamine by microsomes derived from C-ER are consistent with our earlier observation that these membranes have the capacity to synthesize, translocate and process preprolamine (Krishnan and Okita, 1986). These microsomal membranes, however, were significantly less efficient in translocating and processing prepro-

glutelin as compared to preprolamine. The relative inefficient transport and processing of preproglutelin suggests that the wheat germ extract and/or rice microsomal membranes are deficient in one or more cellular factors required for glutelin synthesis. In support of this hypothesis is the finding that the protein translocation efficiency of maize microsomes can be significantly enhanced by the addition of purified 70 kD heat shock protein, a cytosolic protein. Alternatively, differences in the structures of preprolamine and preproglutelin signal peptides may contribute to the difference in translocation efficiencies *in vitro*. DNA sequencing of cDNA clones reveals that preprolamines possess a typical signal peptide structure (Kim and Okita, 1988) whereas the preproglutelin signal peptides contain two cysteines separated by four leucine residues (Okita *et al.*, 1989). These cysteines in the glutelin signal peptides can potentially form a disulfide bond during polypeptide synthesis *in vivo* but such a secondary structure may not be formed under the *in vitro* conditions due to the high levels of reducing agent required by the *in vitro* translation assay.

Lastly, attempts have been made to obtain a fraction enriched for PB-ER to determine whether these membranes are predisposed toward preprolamine synthesis, translocation and processing as suggested by the predominance (5-10 folds) of these transcripts relative to glutelin species on these membranes (Li *et al.*, 1993). At present we have been unable to obtain an enriched PB-ER fraction to carry out these experiments due to substantial contamination of the protein body fraction with large membranous C-ER complexes as well as the fact that the PB-ER constitute only a small percentage of the total membranes of the endosperm cell. Inevitably, studies to address the capacity of these ER types to synthesize specific storage proteins must be conducted in an *in vivo* system, where the membrane targeting of genetically engineered transcripts can be evaluated in transgenic plants.

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(Received June 30, 1994)

벼 胚乳細胞에서 分離된 미크로좀 內로의 貯藏蛋白質 移動

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적 요

발생단계의 벼 배유세포는 cisternae ER(C-ER) 그리고 단백질체 ER(PB-ER) 등 형태상으로 다른 두 개의 조면소포체를 가지고 있는데 그 중 PB-ER은 prolamine 저장단백질체를 둘러싸고 있다. 벼 배유의 mRNAs는 이러한 소포체상에 무작위로 존재하는 것이 아니라 종류에 따른 분포상의 차이를 보이는데, glutelin mRNA는 C-ER에 prolamine mRNA는 PB-CR에 특이하게 존재하고 있음이 밝혀졌다. 벼 배유에서 C-ER이 특이하게 함유된 미크로좀분획을 분리한 후, 이 분획이 저장단백질의 성숙과정 및 막이동과정에 미치는 능력을 *in vitro* translation 방법을 이용하여 조사하였다. C-ER 미크로좀은 *in vitro* translation에 의해 합성된 preproglutelin과 preprolamine 단백질의 신호펩티드를 제거하며 또 이러한 단백질을 미크로좀 소낭내로 이동시키는 능력을 보였다. 특히 preprolamine의 성숙과정과 막이동과정이 C-ER 미크로좀에 의해 보다 효과적으로 진행되었다. 이러한 결과를 통해 C-ER은 저장단백질 합성과정중 저장단백질 mRNA를 특이하게 인식하여 단백질의 성숙 및 막이동과정에 관여함을 알 수 있었다.

주요어 : 벼, 저장단백질, glutelin, prolamine, 막이동

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