Protein translocation across wheat germ microsomal membranes requires an SRP-like component

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Different wheat germ extracts were tested for the presence of membranes capable of translocating and processing nascent secretory proteins. One lysate was found in which nascent prehuman-placental lactogen (phPL) was translocated and processed to mature human placental lactogen (hPL). Processing was found to occur concomitant with translocation across membranes. Translocation across the wheat germ membrane required a component which is similar to the mammalian signal recognition particle (SRP). It bound to DEAE–Sephadex, had a sedimentation coefficient of 11S and contained a 7S RNA. In addition to hPL, the plant protein zein and the bacterial protein β -lactamase were translocated across and processed by wheat germ membranes. Transport was found to occur only co-translationally. Our results show that the wheat germ protein translocation system is similar to the mammalian one. Unlike the mammalian SRP, the particle purified from wheat germ did not arrest elongation of nascent secretory proteins.

Key words: plant membranes/protein translocation/signal recognition particle/wheat germ

Introduction

Significant progress has been made in the elucidation of the mechanism involved in the first steps of protein translocation across the endoplasmic reticulum (ER) membrane in mammalian cells (for reviews, see Walter *et al.*, 1984; Rapoport, 1986). Two components involved in this process, the signal recognition particle (SRP) (Walter and Blobel, 1980) and an integral protein of the ER membrane, the docking protein (DP) (Meyer *et al.*, 1982; Gilmore *et al.*, 1982), have been characterized. They function in the targeting of ribosomes which are synthesizing proteins destined for translocation across the ER membrane. SRP and DP were purified from dog pancreas and their function was tested *in vitro*. In a wheat germ cell-free translation system, canine SRP was shown to cause a site-specific elongation arrest of the synthesis of secretory proteins after the signal sequence had emerged from the ribosome (Walter and Blobel, 1981). DP released the elongation arrest and the nascent protein was translocated across the membrane of the ER (Meyer *et al.*, 1982; Gilmore *et al.*, 1982). When tested in reticulocyte or HeLa cell-free translation systems (Meyer, 1986), DP was still found to be an essential component of the translocation system but no arrest of polypeptide chain elongation was found with canine SRP.

Therefore, it is conceivable that elongation arrest is caused by a mismatch of components from species belonging to different kingdoms. An incompatibility of the components in an heterologous *in vitro* system may also provide a possible explanation for the fact that SRP-depleted mammalian rough membranes are inactive in the wheat germ system.

In order to investigate these possibilities, it is necessary to establish an *in vitro* system in which all the components required for the translation and translocation of proteins are derived from the same source, preferably from wheat germ. An homologous system from *Saccharomyces cerevisiae* has been described recently (Rothblatt and Meyer, 1986; Hansen *et al.*, 1986; Waters and Blobel, 1986), but the mechanism of translocation in yeast is still very poorly characterized and also may be quite different from the one known in higher eukaryotes.

We report here on the development of a homologous wheat germ *in vitro* translation-translocation system. A preliminary characterization of the components involved in the protein translocation process in wheat germ demonstrates that a translocation mechanism similar to the one in mammals also exists in plants.

Results

When messenger RNAs coding for secretory proteins are translated in a wheat germ cell-free system only precursor polypeptides are usually obtained (Figure 1, lane 1). This indicates that a component necessary for processing of secretory proteins is lacking in this *in vitro* system (referred to as wheat germ extract I). However in extracts from a particular source of wheat germ (referred to as wheat germ extract II), we observed that translation of pre-human placental lactogen (phPL) mRNA gave rise to a product co-migrating with the mature form (hPL) of the protein (Figure 1, lane 2). It appeared that this particular wheat germ extract contained an endogenous processing activity present which was able to cleave off the signal peptide. No processed peptides were seen when mRNA was omitted (lanes 3 and 4).

The processing activity seems to reside in a membrane fraction since about 80% of it could be removed from the crude extract by centrifugation at 20 000 g (lane 5). When the sediment of such a centrifugation was added back to another extract which did not show processing activity, conversion of phPL to hPL was observed (lane 6). The extent of processing was dependent on the amount of membrane fraction added and maximally reached about 70% processing (data not shown). The processed hPL was shown to be resistant to the action of proteinase K (Figure 2, lane 2). Simultaneous addition of proteinase K and detergent led to complete degradation of the protein (Figure 2, lane 3). This suggests that hPL was sequestered into membrane vesicles. It should be noted that a polypeptide of intermediate size between phPL and hPL was seen in some experiments, arising apparently from proteolytic degradation of phPL. In contrast to hPL, this

Fig. 1. phPL is proteolytically processed to hPL by membranes from wheat germ. mRNA for phPL was translated in extracts prepared from two different sources of wheat germ, here called extract I and extract II. **Lanes 1 and 3** show the translation in extract I, **lanes 2 and 4** in extract II. Translation was in the presence of phPL mRNA (**lanes 1,2,5 and 6**) or in the absence of mRNA (**lanes 3 and 4**). **Lane 5** shows translation in extract II from which membranes had been removed by sedimentation, **lane 6** shows translation in an extract as used in **lane 5** but with twice the amount of membranes originally present. The final concentration of membranes was 2.5 A_{260} units/ml.

Fig. 2. Translocation of different proteins across wheat germ microsomal membranes. mRNA coding for phPL (**lanes 1–3**), for the plant storage protein zein (pz; **lanes 4 and 5**) or for the bacterial β -lactamase (pbla; **lanes 6 and 7**) were translated either in wheat germ extract I (**lanes 4 and 6**) or the processing wheat germ extract II (**lanes 1,2,3,5 and 7**). After translation, the sample shown in **lane 2** was treated with proteinase K and the one shown in **lane 3** with proteinase K and detergent.

polypeptide was completely digested by proteinase K even in the absence of detergent.

Wheat germ membranes were not only able to process phPL, but also various other precursor proteins including those of the

Fig. 3. Dissection and reconstitution of the translocation activity of wheat germ membranes. Active wheat germ II membranes (RM) were extracted with high salt (500 mM KOAc) to yield K-RM and a salt extract (SE). These components were tested for their ability to reconstitute translocation competence when added to the translocationally inactive wheat germ extract I. phPL mRNA was translated in wheat germ extract I (**lane 1**) and supplemented with RM of wheat germ lysate II (final concentration 4.0 A_{280} units/ml) (**lane 2**) K-RM of wheat germ lysate II (final concentration 4.0 A_{280} units/ml) (**lane 3**) or with K-RM as in **lane 3** and SE derived from 8 A_{280} units of RM of wheat germ lysate II (**lane 4**).

plant protein zein (z; Figure 2, lanes 4 and 5) and the bacterial β -lactamase (bla, lanes 6 and 7). We also have tested γ -interferon and lysozyme, both of which were processed in this manner. Surprisingly, immunoglobulin light chain was not processed at all (data not shown). Processing was always coupled to translocation as evidenced by protease resistance. Whenever signal peptide cleavage was observed, the translocation of a protease-resistant mature protein could be demonstrated. In none of the cases we analysed was translocation observed when membranes were added after translation.

In order to characterize the translocation system of wheat germ in more detail, we followed the protocol which had been used successfully for the dissection of the mammalian endoplasmic reticulum membrane (Warren and Dobberstein, 1978; Walter and Blobel, 1983). The membrane pellet obtained after the 20 000 g centrifugation was extracted with a buffer containing 500 mM KOAc (see Materials and methods). This treatment abolished the translocation activity of the membranes (Figure 3, lane 3). Activity could be regained by adding back the salt extract (Figure 3, lane 4). Various other cell fractions were also tested for their ability to reconstitute translocation activity of salt extracted wheat germ microsomes. A salt extract of a pellet resulting from a 100 000 g centrifugation was found to be even more active than the salt extract which had been derived from the 20 000 g centrifugation.

Aliquots of both salt extracts were extracted with phenol–chloroform and the RNA contained in the aqueous phase was analysed on 7% polyacrylamide gels containing 8 M urea. A small sized RNA which migrated slightly slower than canine 7 SL RNA (Walter and Blobel, 1982) (Figure 4) was detected by ethidium bromide staining. The wheat germ 7S RNA could be labeled with [32 P]pCp and RNA ligase indicating a free 3'-OH end (data not shown). These data provided preliminary evidence that a component in wheat germ exists which might be similar to the mammalian signal recognition particle.

Further support for the assumption that an equivalent of the

Fig. 4. Salt extracts of wheat germ membranes and ribosomes contain a RNA of about 7S. The following RNAs were separated on a 7% polyacrylamide gel containing 8 M urea. **Lane 1:** 7S RNA from dog pancreatic SRP, **lane 2:** RNA from a salt extract of wheat germ RM, **lane 3:** RNA obtained by salt extraction of a wheat germ ribosomal fraction. The arrowhead denotes the position of the canine 7S RNA.

mammalian SRP also exists in plants was provided by the fact that the active component could be bound to DEAE-Sephacrose 6B and could be eluted at a salt concentration of 500 mM KOAc. This behavior is similar to that of mammalian SRP (Walter and Blobel, 1983). After sucrose gradient centrifugation of components eluted from DEAE-Sephacrose 6B at 500 mM KOAc, wheat germ 7S RNA was recovered in fractions corresponding to an 11S particle (Figure 5a, fractions 8,9 and 10). This is the exact size of canine SRP (Walter and Blobel, 1980). The wheat germ particle purified by sucrose gradient was able to reconstitute the translocation competence of salt-extracted microsomes derived from wheat germ (Figure 5b, fractions, 8,9 and 10).

We next tested whether the salt extract from wheat germ ribosomes was able to reconstitute the translocation activity of canine microsomal membranes which had been extracted with 500 mM KOAc (referred to as canine K-RM). Canine K-RM showed some residual processing activity (Figure 6, lanes 1 to 5) which was not significantly enhanced when increasing amounts of the DEAE-Sephacrose bound fraction from the salt extract of wheat germ ribosomes (SE) were added. In contrast, when the same amounts of salt extract were tested with wheat germ K-RM, increased processing of phPL to hPL was observed (Figure 6, lanes 6 to 9). Canine SRP has been shown to arrest further elongation of phPL in the wheat germ cell free system when tested in the absence of membranes (Walter and Blobel, 1981; Bassüner *et al.*, 1984). No such effect was seen when the salt extract of wheat germ ribosomes was added back to a wheat germ translation system (Figure 6, lanes 10–12).

It is known that the arrest of nascent phPL can be released by adding canine K-RM (Walter and Blobel, 1981). When canine SRP was added to a wheat germ translation programmed with phPL mRNA, it strongly inhibited the appearance of phPL (Figure 7, lanes 2 and 3). Addition of salt-washed wheat germ membranes completely overcame this inhibitory effect (compare Figure 7, lanes 3 and 4). This result indicates that canine SRP can functionally interact with wheat germ membranes.

It has been shown previously that the translocation activity of canine K-RM can be inactivated by NEM. This is probably due to inactivation of docking protein (Jackson and Blobel, 1977;

Fig. 5. Sucrose-density gradient centrifugation of the salt extract of wheat germ ribosomal fraction. **(a)** The salt extract from wheat germ ribosomal fraction was bound to DEAE-Sephacrose and the fraction eluted at 500 mM KOAc was layered on top of a sucrose gradient (5 to 20% w/w). After centrifugation in a Beckmann SW40 rotor for 20 h at 20°C the gradient was fractionated. RNA from each fraction was extracted with phenol-chloroform and separated on a 7% polyacrylamide gel containing 8 M urea. Lanes numbered 1 to 11 contain fractions from the top to the bottom of the sucrose gradient. **Lane 12** contains RNA from the pellet of the gradient. **Lane 13** shows the position (marked by the arrowhead) of 7S RNA isolated from canine SRP. Wheat germ 7S RNA is found in fractions 8,9 and 10 corresponding to an 11S particle. **(b)** a 4 μ l aliquot from each of the fractions 4–11 were tested for their ability to reconstitute translocation activity of wheat germ K-RM. Activity is found in fractions 8,9 and 10 as indicated by the processing of phPL to hPL.

Meyer and Dobberstein, 1980). In order to test whether an NEM sensitive component is also required for protein translocation across wheat germ membranes we treated K-RM of wheat germ with NEM. No translocation or processing activity could be observed by addition of canine SRP to the NEM-treated membranes (Figure 7, lane 7). Membranes treated in the absence of NEM were competent in processing phPL when supplemented with canine SRP (compare Figure 7, lanes 5 and 6).

Fig. 6. Functions of a salt extract of wheat germ ribosomal fraction purified on DEAE–Sephadex. A salt extract of wheat germ ribosomes was purified and concentrated on DEAE–Sephadex and was tested for its ability to restore the translocation and processing activity of either canine or wheat germ salt washed membranes (K-RM). mRNA for phPL was translated in the presence of canine K-RM, wheat germ K-RM or DEAE purified wheat germ SRP as indicated above the figure. The intrinsic translation activity is indicated by the translation of globin mRNA (glo) which has been added to the wheat germ extract.

Discussion

Signal sequences have been identified in many plant precursor proteins destined for translocation across rough endoplasmic reticulum membranes (Larkins *et al.*, 1979; Bassünér *et al.*, 1984). However, the protein translocation mechanism in plants is largely unknown. The present study was designed to investigate the presence of a translocation system in plants and ask whether receptors similar to the signal recognition particle (SRP) and docking protein (DP) of the mammalian system are also involved.

Since a function for SRP could only be shown in a heterologous system containing components of plant and mammalian origin, wheat germ appeared to be a good choice for the reconstitution of an homologous *in vitro* translocation system. After screening several batches of wheat germ, one was found that processed precursors to secretory proteins. We speculate that the time of harvest, the storage of wheat and the procedure of germ preparation, among other factors, might determine the activity of a wheat germ translocation and processing system.

In many respects the wheat germ system resembled the mammalian one. We found that several precursors for secretory proteins from plant (zein), mammalian (hPL) or bacterial (β -lactamase) origin could be translocated and processed by wheat germ membranes. Transport was found to occur only co-translationally. For none of the proteins tested could a post-translational transport be found.

A membrane containing fraction that was able to reconstitute translocation activity when added to an inactive wheat germ lysate could be isolated from wheat germ. This activity of the membrane was abolished when the membranes were extracted with salt at a concentration of 500 mM. Activity could be reconstituted by the addition of this salt extract. This is the first homologous *in vitro* protein translocation system in which requirement of a

Fig. 7. Canine SRP (SRP) interacts functionally with salt washed wheat germ membranes (K-RM), but not with those pretreated with NEM. phPL mRNA was translated in wheat germ extract I containing no SRP (lanes 1 and 5), 0.25 μ l SRP (lane 2), 0.5 μ l SRP (lane 3), 1 μ l SRP and K-RM (lane 4 and 6), K-RM (lane 5), and NEM-treated K-RM and 1 μ l SRP (lane 7). Samples 1–4 were separated on a 10–15% polyacrylamide gel. Samples 5–7 were separated on a 10% polyacrylamide gel.

salt extractable component for protein translocation has been shown. In an homologous mammalian system no such dependence on an exogenous salt extracted component has been directly demonstrated. It has been proposed that the observed arrest by SRP is a result of the heterologous nature of the components used in the *in vitro* reconstitution experiments, namely, SRP from canine pancreas and ribosomes and translation factors from wheat germ (Meyer, 1986). Our results clearly demonstrate that SRP is also required in an homologous translation-translocation system. A ribonucleoprotein particle could be extracted from wheat germ membranes and ribosomes which is very similar to the mammalian signal recognition particle. It has a sedimentation constant of 11S, contains a 7S RNA, is salt extractable from the pellet of a centrifugation at 20 000 g and is required for protein translocation across K-RM membranes. Not only this particle (tentatively called wheat germ SRP), but also a proteinaceous membrane receptor similar to the mammalian DP seems to exist in wheat germ. When K-RM membranes of wheat germ were treated with NEM no activity could be reconstituted by wheat germ SRP. The DP has been shown to be an NEM sensitive component of canine rough microsomes (Jackson and Blobel, 1977; Meyer and Dobberstein, 1980).

Despite the common features of the plant and the mammalian translocation systems, mammalian and plant components cannot substitute each other in all cases. Canine SRP can interact functionally with wheat germ microsomes, but wheat germ SRP does not reconstitute translocation function with mammalian K-RM. Possibly these results reflect quantitative differences in the binding constants of the two SRPs to the nascent polypeptide chain or to the respective membrane receptors. Up until now elongation arrest could only be demonstrated in a system where canine SRP was tested in a wheat germ cell-free extract. The wheat germ SRP did not show any inhibition effect on elongation of secretory proteins at all concentrations tested. To the contrary, addition of the SRP containing fraction consistently increased the efficiency of translation. Since our wheat germ SRP preparation is not pure, we cannot exclude that initiation, elongation or some other stimulating factors co-purify with the wheat germ SRP.

It is interesting to note that most of the functionally active ribonucleoprotein component of wheat germ is retained at low salt concentrations in the ribosomal fraction. It cannot be decided whether the wheat germ particle is bound directly to ribosomes or whether it is associated with other components like small membrane vesicles. The only other homologous translation-translocation system established so far is that of *S. cerevisiae* (Rothblatt and Meyer, 1986; Hansen *et al.*, 1986; Waters and Blobel, 1986). However, there is no evidence as yet for a SRP-like factor in *S. cerevisiae*. This suggests that the wheat germ protein translocation system is more similar to the mammalian system than to that of *S. cerevisiae*.

Materials and methods

Isolation of messenger RNAs

Human pre-placental lactogen (phPL) mRNA was isolated from placenta and purified by poly-U-Sepharose chromatography (Prehn *et al.*, 1981). Immunoglobulin light chain mRNA was isolated from MOPC 41 myeloma cells (Blobel and Dobberstein, 1975). Messenger RNA for β -lactamase was obtained by *in vitro* transcription of plasmid pTD 101 (Date and Wickner, 1981) and zein mRNA was derived from plasmid pDS-M1 Hpa, Dra (Spena *et al.*, 1985).

Cell-free synthesis in a wheat germ system

The conditions for the cell-free synthesis in a wheat germ system were essentially those of Roberts and Patterson (1973). The *in vitro* translation-translocation assay contained in a final volume of 25 μ l: 5 μ l of 16 000 g wheat germ supernatant, 1 μ l of [35 S]methionine (800 Ci/mmol) and 0.1 to 0.3 μ g mRNA. The final concentrations of other substances were: 110 mM KOAc, 3 mM

Mg(OAc) $_2$, 2 mM DTT, 8 mM creatine phosphate, 1.2 mM ATP, 0.25 mM GTP, 75 μ g/ml creatine phosphate kinase and 23 μ M amino acids (except methionine). Incubation was carried out for 60 min at 25°C.

Preparation of rough microsomes (RM) and SRP from dog pancreas were carried out according to the protocol of Walter and Blobel (1983). NEM-treatment of membranes was carried out according to Gilmore *et al.* (1982).

Translation products were analysed by SDS-gel electrophoresis according to Laemmli (1970). Polyacrylamide gels with a 10–15% linear gradient were used with a 5% stacking gel. In some cases 10% polyacrylamide gels were used. Gels were processed for fluorography as described by Bonner and Laskey (1974) and exposed to X-ray films (Fuji, RX 100) at –70°C.

To study the protection of processed proteins after their *in vitro* synthesis, one third of the sample was treated with proteinase K (final concentration 0.3 mg/ml) for 45 min at 0°C. Another aliquot of the sample was treated in parallel, except that Nonidet P-40 (Sigma) was added to a concentration of 0.5%.

Phenol-chloroform extracted RNA from the various fractions was analysed by electrophoresis on 1 mm thick 7% polyacrylamide gels (acrylamide:bisacrylamide 19:1) in 90 mM Tris-borate pH, 2.5 mM EDTA containing 8 M urea and staining with ethidium bromide.

Preparation and fractionation of a wheat germ lysate

Wheat germ was ground in a mortar in liquid nitrogen to yield a fine powder. Extraction was carried out with 20 volumes of homogenization buffer (50 mM TEA, pH 7.5; 40 mM KOAc, 2 mM Mg(OAc) $_2$, 5 mM DTT, 10 μ g PMSF/ml, 250 mM sucrose) for 10 min. at 0°C. The homogenate was centrifuged for 10 min. at 8 000 g. A second centrifugation at 20 000 g for 40 min. yielded a pellet which was resuspended in a buffer (50 mM KOAc, 2 mM Mg(OAc) $_2$, 1 mM DTT, 50 mM TEA, pH 7.5, 250 mM sucrose) to a final concentration of 25 A $_{260}$ -units/ml. The supernatant was subjected to ultracentrifugation (2.5 h, 120 000 g) to yield a pellet (containing mostly ribosomes) which was dissolved in the same buffer (see above).

For the preparation of the active ribonucleoprotein fraction, the dissolved pellet of the 20 000 g centrifugation as well as the ribosomal pellet were adjusted to 50 mM TEA, pH 7.5, 500 mM KOAc, 5 mM Mg(OAc) $_2$ and 2 mM DTT and pelleted again by centrifugation for 2.5 h at 120 000 g. The salt extract was diluted with 2 vol of water and applied onto a DEAE-Sepharose-column and bound material was eluted with buffer containing 50 mM TEA pH 7.5, 500 mM KOAc, 5 mM Mg(OAc) $_2$, and 2 mM DTT. The eluate was adjusted to a final concentration of 2.0–2.5 A $_{260}$ -units/ml, frozen in liquid nitrogen and stored at –70°C.

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