Sheep pancreatic microsomes as an alternative to the dog source for studying protein translocation

Mustak A. KADERBHAI,*† Victoria J. HARDING,* Amna KARIM,*§ Brian M. AUSTEN† and Naheed N. KADERBHAI*

*Institute of Biological Sciences, University of Wales, Penglais, Aberystwyth, Dyfed SY23 3DD, Wales, and †Surgical Unit, St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, U.K.

A procedure is described for the preparation of rough membrane vesicles of endoplasmic-reticular origin from the pancreas of sheep. These isolated membranes translocate, process and glyco-

sylate *in vitro*-translated heterologous proteins in a manner comparable with that exhibited by dog pancreatic microsomes.

INTRODUCTION

Our understanding of the movement of proteins to and across the endoplasmic-reticulum (ER) membrane and the associated modifications has arrived from the use of heterologous cell-free translation systems programmed with mRNAs in the presence of isolated microsomes [1,2]. The most extensively used membrane system for *in vitro* protein-transport studies is the one derived from dog pancreatic rough ER [3], although those from rat liver [4], yeast [5,6], hen oviduct [7] and other sources [8,9] have also proved successful. The ease of isolation of translocation-competent rough-ER-derived membranes (RM) from dog pancreas is thought to be attributable to (i) the abundance of RM in this particular organ and (ii) the high level of endogenous RNAase inhibitor which preserves the integrity of polysomes bound to microsomes during their isolation. Over the last decade, the dog microsome has become a well-established and dependable membrane system for studying protein transport and the associated complex co- and post-translational modifications. Nevertheless, an alternative source could prove more convenient and also provide opportunities to investigate the universal features of protein translocation across the ER. Previous attempts to isolate protein-translocation-competent RM from the pancreas of pig, rat and fish have proved unsuccessful (M. A. Kaderbhai and B. M. Austen, unpublished work). The present paper describes some of the characteristics of the abundantly isolatable sheep pancreatic RM in their ability to translocate and modify pre-proteins.

MATERIALS AND METHODS

Isolation of membranes

RM were prepared from sheep pancreas (approx. 30 g) essentially as described by Kaderbhai and Austen [10]. Ewes were given (or not) 20 units of pancreozymin/kg body weight prior to excision of the pancreas. All subsequent operations were conducted at 4 °C. After dissecting away the connective tissue, the organ was extensively washed with chilled homogenizing (H) buffer, to which phenylmethanesulphonyl fluoride at 2.5 mM and aprotinin at 1000 units/ml were added immediately prior to use. The tissue, finely minced using a hand tissue press, was mechanically homogenized in H buffer (1 g of tissue/ml) by applying ten

complete passes. A postmitochondrial supernatant obtained by centrifugation of the homogenate at 14 500 g was applied to a Sepharose 4B column (5 cm int. diam. × 12 cm high), and the membranes, resolved from the soluble fraction, were collected in the void elution volume. A fraction (10 ml) was applied on to a discontinuous sucrose density gradient consisting of 7.5 ml of 1.5 M sucrose in 50 mM Tris/HCl (pH 7.4)/25 mM KCl/5 mM MgCl₂ (TKM), 7.5 ml of 1.75 M sucrose in TKM and 7.5 ml of 2.1 M sucrose in TKM and centrifuged at 105 000 g for 16 h. RM were harvested from the 2.1 M/1.75 M sucrose interface and diluted with three volumes of TKM. The re-isolated membranes (105 000 g for 1.5 h) were finally suspended by gentle homogenization in 1 ml of ice-cold 25 mM Hepes/KOH (pH 8.0)/20 % (w/v) glycerol, divided into 100 ml aliquots, flash-frozen in liquid N₂ and stored at –80 °C.

For their stripping, freshly prepared RM suspended in 1.65 M sucrose in TKM were gently mixed with 3 vol. of 0.7 M KCl/25 mM Hepes/KOH (pH 7.4)/15 mM EDTA and incubated for 20 min at 0 °C. The salt-treated membranes were layered over a 0.75 M sucrose cushion containing 0.5 M KCl/20 mM Hepes/KOH (pH 7.4) and centrifuged at 105 000 g for 1.5 h at 4 °C. The resulting stripped membranes (SM), found suspended in the sucrose cushion, were harvested, diluted 3-fold with 20 mM Hepes/KOH, pH 7.4, re-centrifuged, and the pellet finally suspended as described above for the RM.

Protein and membrane concentration

The protein content was estimated as described by Bradford [11] using the Bio-Rad (Hemel Hempstead, Herts., U.K.) Coomassie Blue kit. The absorbance at 260 nm of 1 %-SDS-solubilized membranes also served as an index of the measure of membrane concentration.

Translation and translocation

In vitro protein synthesis, unless stated otherwise, was performed in a 10 µl final volume containing 50 % (v/v) micrococcal nuclease-treated TNT rabbit reticulocyte lysate, 4 % (v/v) TNT buffer (Promega, Southampton, U.K.), 4 kunits of RNAase

Abbreviations used: ER, endoplasmic reticulum; α -F, α -factor; H buffer, homogenizing buffer [0.25 M sucrose/50 mM Tris/HCl (pH 7.4)/25 mM KCl/5 mM MgCl₂/2 mM dithiothreitol]; β -L, β -lactamase; pre- β -L, pre- β -lactamase; P- α -F, pro- α -factor; pre-P- α -F, prepro- α -factor; GFP, major glycosylated P- α -F; RM, rough microsomes; SM, stripped microsomes; SRP, signal-recognition particle; TKM, 50 mM Tris/HCl (pH 7.5)/25 mM KCl/5 mM MgCl₂.

† To whom correspondence should be sent.

§ Present address: Department of Biochemistry, Faculty of Medicine, King Abdul Aziz University, Jeddah, Saudi Arabia.

inhibitor/ml, 0.1 mM each of 19 unlabelled amino acids without methionine, 7.5 GBq (4.6 TBq/mmol) of [35 S]methionine/ml, in either the absence or presence of a specified amount of sheep pancreas RM/SM for 60 min at 30 °C. Translation was initiated by adding 50 ng of either *Escherichia coli* pre- β -lactamase (pre- β -L) or *Saccharomyces cerevisiae* prepro- α -factor (pre-P- α -F) mRNA. The amount of radioactivity incorporated into newly synthesized proteins was determined by trichloroacetic acid precipitation using duplicate volumes of 1 μ l of the translation mixtures. Translation mixtures, after boiling with 3 vol. of SDS buffer [5% (w/v) SDS/20% (v/v) glycerol/10 mM Tris/HCl (pH 6.8)/0.003% Bromophenol Blue], were applied (20 000 c.p.m./track) for electrophoresis in the standard 16%-polyacrylamide gels and the resolved bands detected by fluorography [10]. The molecular masses of the radiolabelled proteins were estimated by tracing the positions of the marker proteins (BSA, 68 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; and trypsin inhibitor, 20 kDa) separated in corresponding Coomassie Blue-stained gels.

Signal peptidase assay

The assays were conducted in a final volume of 25 μ l containing 1 mg of purified *E. coli* signal (leader) peptidase, 0.4% (w/v) Triton X-100, 50 mM Tris/HCl, pH 7.4, and 5 μ l of wheat-germ translation system containing [35 S]methionine-labelled pre-P- α -F. Incubation was for 3 h at 35 °C.

RESULTS AND DISCUSSION

Under the electron microscope the sheep RM were observed as intact vesicles ranging in diameter from 50 to 500 nm (results not shown). Morphologically the RM vesicles were typically granular, with a large number of ribosomes attached to their cytoplasmic surface, showing that their native orientation was retained during the isolation processes. That the isolated RM was of a high purity was substantiated by their homogeneous nature and the absence of other cellular organelles in the preparations. RM were obtained in good yields (25–50 mg of protein) from sheep pancreas (30–40 g), a recovery sufficient for over 1000 translation events, each of which requires 2.5–10 mg of protein equivalent.

Co-translational addition of sheep pancreas RM to newly synthesized *E. coli* pre- β -L resulted in conversion of the 31 kDa

Figure 2 Processed β -L is translocated

Post-translation mixtures were incubated without or with trypsin in the absence or presence of 1% (w/v) sodium deoxycholate on ice for 1 h. Proteolysis was inactivated by addition of 5.5 kunits of aprotinin/ml and 2.5 mM phenylmethanesulphonyl fluoride with 30 min incubation at 0 °C.

precursor to a processed band of 29 kDa (Figure 1a), presumably β -lactamase (β -L). The extent of processing gradually increased with the accretion of the RM (Figure 1b), although a gradually increasing inhibition of translation was apparent with use of RM at or above 11.2 A_{260} units (Figure 1a, tracks 5–10). Unlike the canine RM [20], the sheep pancreas RM did not require nuclease treatment and gave little or no background protein synthesis from endogenous mRNA (tracks 11–15, Figure 1a).

Processing of nascent preproteins targeted to microsomal vesicles occurs only if the protein is translocated across the microsomal membranes, as the active site of signal peptidase is confined to the luminal surface [3]. Post-translational limited proteolyses were performed to ascertain that the processed pre- β -L was internalized into the vesicles (Figure 2). Exogenous trypsin at 50 μ g/ml or above (Figure 2, tracks 5 and 6) completely degraded pre- β -L, but not β -L. This showed that the processed counterpart must have been internalized into vesicles to be afforded protection against the extravesicular proteinase. The vesicularized product, however, was sensitive to degradation when the membrane barrier was disrupted by the addition of sodium deoxycholate (Figure 2, track 7).

Figure 1 Pre- β -L is processed by sheep RM

Translations were performed as described in the Materials and methods section with *E. coli* β -L mRNA either in the absence or in the presence of increasing amounts of RM. (a) Fluorogram; (b) extent of cleavage quantified from pre- β -L and β -L bands. Abbreviation in this and other Figures: M, molecular mass.

Figure 3 Consensus signal peptide (CSP) inhibits pre- β -L translocation into-sheep microsomes

CSP, consensus signal peptide; PTH²⁴⁻²⁸, parathyroid hormone-(24-28)-peptide; TP¹⁻²³, 1-23-residue transit peptide of wheat ribulose-1,5-bisphosphate carboxylase small subunit.

A 19-residue synthetic consensus signal peptide has previously been demonstrated to arrest competitively the translocation and processing of several natural pre-proteins across the membranes of dog pancreatic RM [12]. The consensus peptide, after pre-incubation with sheep RM, caused a dose-dependent inhibition of pre- β -L processing in the transport assays (Figure 3, tracks 1-6); almost complete inhibition of the processing/translocation was reached at 40 μ M peptide (Figure 3, track 6). Comparable inhibition was provoked neither by the 28-48-residue segment of the parathyroid hormone (Figure 3, tracks 7 and 8), a peptide of size and hydrophobicity index comparable with that of the consensus peptide, nor by a water-soluble 1-23-residue chloroplast-targeting transit peptide of the small subunit of wheat ribulose-1,5-bisphosphate carboxylase (Figure 3, tracks 9 and 10). This specific inhibition by the consensus signal peptide demonstrates that the recognition of the pre- β -L signal sequence by the RM is an essential feature of the ovine translocation machinery which appears to function in a manner comparable with that established in the dog pancreatic RM [13].

Signal-recognition particle (SRP), purified from dog pancreatic RM [14], is a complex of six distinct proteins in association with a 7 S RNA which selectively recognizes polysomes engaged in the synthesis of secretory or membrane-spanning proteins by interacting with the signal or stop-transfer sequence after its emergence from the ribosomes [15,16] and imposes site-specific transient inhibition of polypeptide-chain elongation. The interaction of the translation-arrested ribosome-SRP complex with the SRP receptor (docking protein) in the ER reinstates polypeptide-chain elongation, thus initiating the feature of co-translational protein translocation across the membrane. In the cell SRP is evenly distributed between the cytosol, rough ER and polysomes [17], and therefore it would be expected to be recovered in SM, isolatable after treatment of RM with high salt concentration. The question thus arises whether sheep SRP was involved in targetting and translocation, particularly so in the light of the possibility that the prevailing activity of SRP in the rabbit reticulocyte lysate, deployed in the import assay, may have masked the active or inactive particle derived from the ovine membranes. The co-translational translocation activity of SRP present in microsomes can be assessed in a wheat-germ translation system, which, for as yet unknown reason(s), possesses an SRP-related translocation component(s) that is either disabled or incompatible with its mammalian counterpart [18]. The synthesis

Figure 4 SRP-dependency of pre- β -L targetting to microsomes

Translations were performed in micrococcal-nuclease-treated wheat-germ lysate as described in the Materials and methods section, in either the presence or absence of RM or SM. Indicated amounts of high-speed reticulocyte-lysate supernatant fraction were included to supplement SRP.

of pre- β -L in a wheat-germ translation system coupled with sheep RM resulted in only a marginal processing, of about 8% (Figure 4, track 6). Despite the fact that this level of processing is some 10-fold poorer than that obtained with a comparative amount of RM in the reticulocyte translation system (Figure 1a, track 3), it is indicative of the presence of an active SRP pool in the isolated sheep RM. Further supplementation of SRP, by addition of 230 000 $g \cdot h$ reticulocyte-lysate supernatant fraction to the wheat-germ system, imposed a dose-dependent inhibition of precursor formation, but without influencing the final level of processing (Figure 4, tracks 7-9), implying that, unlike the mammalian translation system, the wheat extract exerts a limit on the rate of either targetting to, or translocation into, microsomes of the nascent precursor arriving from the SRP-ribosome-peptidyl complex. In contrast with RM, the use of SM in the wheat-germ translation system annulled pre- β -L processing (Figure 4, track 2). This is consistent with the notion that, in the absence of SRP, nascent precursor proteins do not encounter their transient translation arrest and so the fully synthesized polypeptides are neither targetted to the microsomal surface nor cleaved. SRP supplementation from a high-speed reticulocyte-lysate fraction restored the processing activity of SM to a level comparable with that observed in RM (tracks 3-5, Figure 4), although it induced gradual inhibition of the precursor formation. These findings clearly substantiate the presence and participation of SRP derived from sheep RM in the co-translational processing of pre- β -L.

α -F of *S. cerevisiae* is synthesized as a larger form, pre-P- α -F, which undergoes a series of processing events in the secretory pathway, described by Palade [19], eventually culminating into four copies of a 13-residue mature secretory pheromone [21]. The 165-amino-acid α -F precursor contains at its N-terminus a 19-residue cleavable signal sequence and three Asn sites in the 'pro' region for core glycosylation in the ER lumen [22]. *In vitro* translation of pre-P- α -F mRNA directed synthesis of a 20 kDa protein which is clearly of the expected size of pre-P- α -F, in agreement with previous reports [21-24] (Figures 5a and 5b, track 1). Incubation of sheep pancreas RM during *in vitro* protein synthesis converted the precursor into a significantly slower moving band of about 30 kDa (Figures 5a and 5b, tracks 3-9); the level of conversion into the 30 kDa band closely corresponded to the amount of RM added in the translation

Figure 5 Processing of pre-P-α-F

Pre-P-α-F mRNA was translated in the absence or presence of various amounts of sheep RM. The processing of pre-P-α-F by purified *E. coli* signal peptidase was conducted post-translationally as described in the Materials and methods section. (a) and (b) show electrophoretic separations without and with 4 M urea respectively in 16%-polyacrylamide gels. (c) Formation of α-F derivatives during translocation. Abbreviation: GPF, major glycosylated form of P-α-F.

system (Figure 5c). The facts that the 30 kDa product (i) after deglycosylation with endoglycosidase F (Boehringer Mannheim) can be converted into a form having a mobility similar to that of the predicted size of the processed pro-α-factor (P-α-F) (Figure 6), and (ii) was protected after trypsin treatment of RM (Figure 7, tracks 3–4), are indicative of the glycosylated nature of the P-α-F that was translocated into microsomal vesicles. It is probable that the two additional, fainter, intermediate bands represent species containing 1 and 2 Asn-linked oligosaccharides each. The present findings with ovine RM are in agreement with those of Waters and co-workers [23], who reported that the higher-molecular-mass product derived from pre-P-α-F after its luminal

Figure 6 Deglycosylation of P-α-F

Microsomal synthesized pre-P-α-F translation mixture (track 2) after solubilization in 0.1% SDS and treatment with Nonidet P40 [0.5% (w/v) final concn.] was incubated without (track 3) or with (tracks 4 and 5) endoglycosidase F (Endo F) for 16 h at 30 °C. Following precipitation with 10% (w/v) trichloroacetic acid, the proteins were solubilized in SDS buffer and analysed in a 16%-polyacrylamide gel. Abbreviation: GPF, major glycosylated form of P-α-F.

internalization into yeast microsomes was due to extensive N-core glycosylation of the translocated P-α-F at the Asn residues. As has been reported previously [23,24], we find that the processed, unglycosylated, pro-α-F separates anomalously with the same mobility as the unglycosylated precursor in our standard 16%-polyacrylamide-gel system, but can be resolved as a slightly faster-moving band than pre-P-α-F when urea at 4 M was included in the separating gel (Figure 5b, track 2); the resistance of P-α-F, but not pre-P-α-F, to degradation by exogenous trypsin (Figure 7) is indicative of its localization into the microsomal lumen. Importantly, the electrophoretically monitored cleavage of post-translationally synthesized pre-P-α-F by purified *E. coli* signal (leader) peptidase was similar to that displayed by sheep microsomes (Figure 5b, track 2), findings which are consistent with the highly conserved reaction specificity of prokaryote and eukaryotic signal peptidases for heterologous precursor substrates [25]. An indication of the sequence of modification events during translocation of pre-P-α-F into the microsomes can be gauged from semi-quantitative analyses of the products trapped at varying doses of the membranes (Figure 5c). From the reciprocal presence of a constant, small pool of

Figure 7 Processed and glycosylated P-α-F forms are translocated

Post-translational proteolysis was performed as described in Figure 2 and the proteins were resolved in a 16%-polyacrylamide gel containing 4 M urea. Abbreviation: GPF, major glycosylated form of P-α-F.

Figure 8 Translocation of pre-P- α -F is co-translational

Sheep RM ($A_{260} = 13.5$) were added at the indicated times after initiation of translation with pre-P- α -F mRNA. The extent of processing was determined from the amount of radioactivity in the major glycosylated form of P- α -F (GPF) and P- α -F bands.

unglycosylated, signal-sequence-processed intermediate versus the absence of an undetectable glycosylated precursor it can be deduced that core glycosylation probably occurs only after the precursor has been fully translocated and processed. The observation that, at the higher membrane concentrations, most of the luminal P- α -F was rapidly converted into the 30 kDa glycosylated form suggests an ample pool of dolichol-linked oligosaccharide in the isolated sheep RM, although the details of immediate modifications after core glycosylation remain to be elucidated. To investigate whether translocation of pre-P- α -F into the sheep pancreatic RM was co- or post-translational, the effect of adding the membranes, at extended durations following initiation of translation, on the productivity of the glycosylated form was measured (Figure 8). Translocation of the pre-P- α -F, although not synchronized, occurred only if the membranes were added within the first 5 min of translation. This implies that, for effective translocation of the nascent chain carrying the exposed signal, co-translational interaction with the RM is equally important.

In this study we have shown that the pancreas of sheep, a herbivore, provides a convenient and an abundant source of RM. Pretreatment of sheep with pancreozymin, to deplete the pool of digestive enzymes in the secretory granules, is not

necessary to obtain intact, functional RM from the organ. In this respect it is possible to use sheep pancreases obtained from a slaughterhouse to isolate the membranes. However, our experiences suggest it is important to initiate isolation of microsomes from a retrieved pancreas with a minimum possible delays in view of the fact that this organ tends to deteriorate rapidly after cessation of the circulation. The membranes are isolatable with considerable ease and remain translocation-active for an indefinite period when stored at -80°C . We have found no need to strip the ribosomes or deplete the endogenous RNAs by nuclease treatment prior to their use in translations with exogenous transcripts. These isolated sheep RM are comparable in their properties with dog pancreas microsomal membranes in that they co-translationally translocate, process and glycosylate heterologous precursor proteins in the standard *in vitro* translation systems.

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