FUNCTIONAL INTERACTION OF PLANT RIBOSOMES WITH ANIMAL MICROSOMAL MEMBRANES

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SUMMARY: Translation of mRNA for the light chain of murine immunoglobulin in a wheat germ cell-free system in the presence of stripped microsomal membranes from canine pancreas resulted in co-translational proteolytic conversion of the precursor of the light chain, reducing it to the size of the authentic light chain of immunoglobulin, and in co-translational segregation of the processed chains in a proteolysis resistant space of the heterologous microsomal vesicles.

In the recently formulated signal hypothesis (1) it has been proposed that an amino terminal extension ("signal sequence") common to all nascent secretory proteins triggers coordinated binding of the ribosome to the membrane and thereby provides the topological conditions for a co-translational transfer of the nascent chain into the intracisternal space of the ER and a co-translational removal of the signal sequence ("processing") by a membrane-bound signal peptidase. Reconstitution experiments using heterologous components such as rabbit reticulocyte ribosomes and "stripped" dog pancreas microsomal membranes have provided evidence that processing of nascent presecretory polypeptide chains (light chain of murine immunoglobulin) and segregation of processed nascent chains within heterologous microsomal vesicles can be achieved in vitro (2). These data suggested that there is a widespread equivalence of sites.

In this present study we report that plant ribosomes (wheat germ) and animal microsomal membranes (dog pancreas) can interact in vitro to result in a co-translational processing of heterologous nascent chains as well as co-translational segregation of the processed chains in heterologous microsomal vesicles. A preliminary account of this work has been presented elsewhere (3).

Abbreviations: SDS, sodium dodecyl sulfate
PAGE, polyacrylamide gel electrophoresis
AR, autoradiography

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MATERIALS AND METHODS: Preparation of a wheat germ extract: A wheat germ 23,000 x g supernate (S 23) was prepared by the method of Roman et al.(4). Briefly, 1.5 g of wheat germ was ground in a chilled mortar in 10 ml of 90 mM KCl, 2 mM $CaCl_2$ and 1 mM Mg $(CH_3COO)_2$. The homogenate was centrifuged at 23,000 x g for 10 min at 4°C and the supernate was removed, avoiding the surface layer of fat. Tris·CH_3COOH, pH 7.6, and $Mg(CH_3COO)_2$ was added to the supernatant to final concentrations of 20 mM and 2 mM, respectively. This was followed by another centrifugation at 23,000 x g for 10 min. The resulting supernate was applied to a Sephadex G-25 column equilibrated in 1 mM Tris·CH_3COOH, pH 7.6, 50 mM KCl, 1 mM $Mg(CH_3COO)_2$ and 4 mM 2-mercaptoethanol. The voided material was collected and centrifuged at 23,000 x g for 10 min. The resulting supernatant--referred to as S 23--was kept frozen in small aliquots at -80°C. It contained 40-60 A_{260} per ml. No loss of activity was detectable after storage of up to three months.

Cell-free protein synthesis: A 100 µl reaction mixture contained the following components: 20-40 µl of S 23, 2.0 µl HEPES·KOH, pH 7.5, at 20°C, 2.0 µmol KCl, 11.0 µmol KCH_3COO, 0.2 µmol $Mg(CH_3COO)_2$, 8 nmol spermine HCl, pH 7.5, 0.2 µmol dithiothreitol, 0.1 µmol ATP, 0.01 µmol GTP, 0.8 µmol creatine phosphate, 4 µg creatine phosphokinase, 15 µCi [^{35}S] methionine (spec. act. 200 Ci/mmol), 2 nmol each of 19 unlabeled amino acids; when indicated in the figure legends, 100 µl of reaction mixture also contained 0.1 A_{260} poly(A) containing RNA (1) isolated from rough microsomes of murine myeloma MOPC 41 DL-1 (1) and various amounts of EDTA-stripped microsomal membranes (RM·EDTA) from dog pancreas (2). Incubation was at 27°C. 10 µl aliquots were removed at indicated time intervals and spotted on 3M Whatman filter paper disks, which were processed according to Mans and Novelli (5). Radioactivity was determined in toluene-Liquifluor.

Post-translational assays: Following incubation for 90 min of the reaction mixture described above, after which no further amino acid incorporation into acid precipitable radioactivity was observed, the following assays were performed. (1) Incubation with membranes: RM·EDTA in amounts specified in figure legends were added to 25 µl of a "post-translational" reaction mixture and incubation was continued at 27°C for 90 min. Incubation was terminated by the addition of 1 vol. ice-cold 10% TCA.
(2) Proteolysis: 25 µl aliquots of the "post-translational" reaction mixture were incubated at 27°C for 1 hr with 5 µl of a solution containing trypsin and chymotrypsin (3 mg of each per ml). Proteolysis was terminated by the addition of 1 vol. of ice-cold 10% TCA.
(3) Immunoprecipitation: 100 µl of the post-translational reaction mixture was adjusted to 1% Triton X-100 and centrifuged at 100,000 x g av for 1 hr. To the resulting supernatant, stock solutions of 1.0 M Tris·HCl, pH 8.6,and 2.0 M KCl were added to give final concentrations of 0.1 M and 0.8 M, respectively. This was followed by the addition of an $(NH_4)SO_4$ fractionated goat antiserum to mouse IgG, an incubation at 37°C for 1 hr, the addition of an $(NH_4)SO_4$ fractionated rabbit antiserum to goat IgG, an incubation at 4°C for 24-48 hr and centrifugation of the immunoprecipitate at 1,000 x g for 5 min. The immunoprecipitate was washed four times by a resuspension and centrifugation cycle: three times in 1 ml 0.01 Tris·HCl, pH 8.6, 0.5 M KCl and 1% (w/v) Triton X-100 and finally once in 1 ml 0.01 M Tris·HCl, pH 8.6. The washed immunoprecipitate was prepared for SDS-PAGE by a procedure identical to that used for TCA precipitates. An aliquot equivalent to 25 µl of post-translational mixture was applied to a slot of the slab gel.

Polyacrylamide gel electrophoresis (PAGE) in sodium dodecyl sulfate (SDS) and autoradiography (AR): TCA- or immunoprecipitates were reduced and alkylated as described (1). Electrophoresis of the reduced and alkylated samples in polyacrylamide gradient slab gels in sodium dodecyl sulfate and subsequent autoradiography of the dried slab gels was as described (1).

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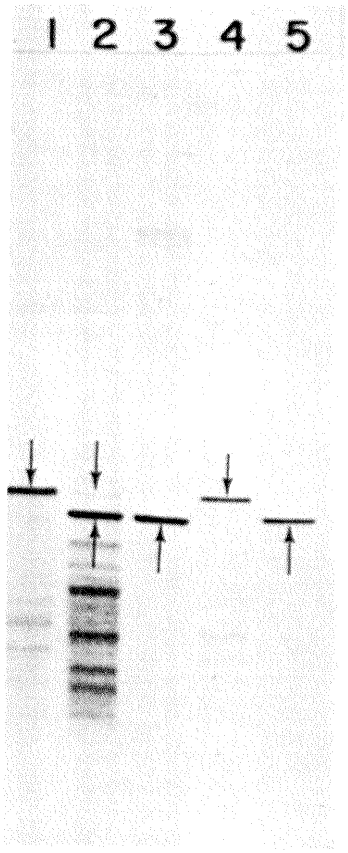

Fig. 1 Analysis by SDS-PAGE and AR of translation products and of their immunoprecipitates.

After translation of mRNA in the cell-free system for 90 min in the presence or absence of membranes (RM·EDTA), 25 µl aliquots were prepared for SDS-PAGE either directly (tracks 1 and 2) or were first subjected to immunoprecipitation (tracks 4 and 5).

Incubation in the cell-free system was as follows: tracks 1 and 4, with mRNA; tracks 2 and 5, with mRNA and with RM·EDTA (4.0 A_{260}/ml). Track 3, radiolabeled secreted light chain of IgG.

Downward and upward pointing arrows indicate the precursor and the processed or secreted IgG light chain, respectively.

Radiolabeled light chains of murine IgG from MOPC 41 DL-1 tumors were prepared as described previously (1). Wheat germ was a generous gift from Mr. Malehot of General Mills, Minneapolis, Minnesota. [^{35}S] methionine (200 Ci/mmol) was obtained from Amersham/Searle Corp., Arlington Heights, Illinois. Goat antiserum to mouse IgG and rabbit antiserum to goat IgG were purchased from Miles Laboratories, Inc., Elkhart, Indiana. The sources of all other materials have been given previously (1).

RESULTS: Polyadenylated mRNA from murine myeloma MOPC 41-DL 1 (1) containing mRNA for the light chain of immunoglobulin was translated in the

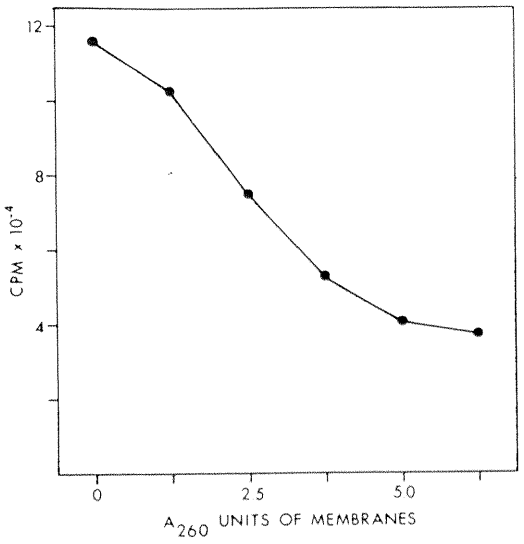

Fig. 2 Inhibition of amino acid incorporation by increasing amounts of membranes.

After incubation of the cell-free system for 90 min in the presence of mRNA and increasing amounts of RM·EDTA per ml of cell-free protein synthesis mixture, radioactivity was determined from 10 µl aliquots (see Methods).

wheat germ cell-free system either in the absence or the presence of dog pancreas microsomal membranes. Analysis of the translation products by SDS-PAGE and AR is shown in Fig. 1. Translation in the absence of membranes (track 1) yielded a prominent band which was identified as light chain "precursor" (6, 7) by antibody precipitation (track 4). Translation in the presence of membranes (track 2) resulted not only in a new band with a mobility identical to that of the secreted light chain of IgG (track 3) and identified as such by antibody precipitation (track 5), but also in a concomitant almost complete disappearance of the precursor band. These data indicated that the co-translational presence of membranes resulted in "processing" of the precursor of the light chain reducing it to the size of the "authentic" light chain of IgG.

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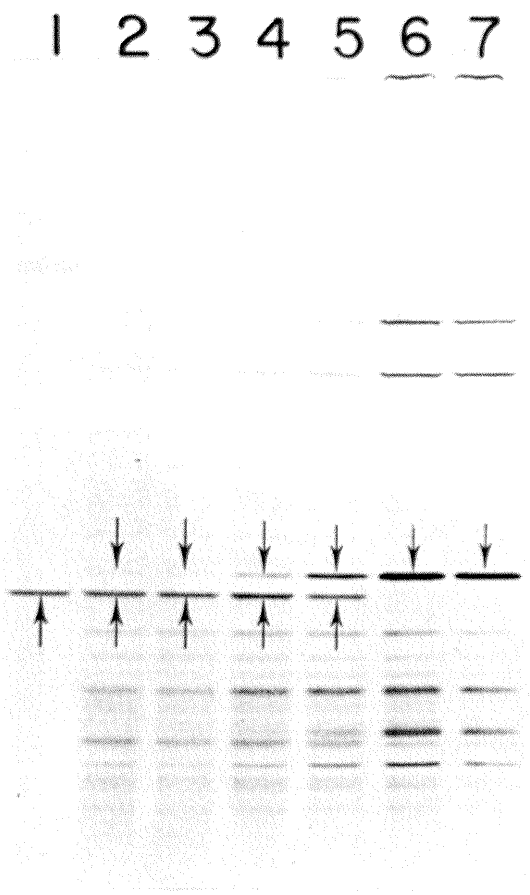

Fig. 3 Analysis by SDS-PAGE and AR of translation products synthesized at 90 min time points as described in Fig. 2. Track 1, radiolabeled secreted light chain of IgG; products synthesized either in the absence (track 7) or in the presence (tracks 2-6) of various amounts of RM·EDTA: 6.75 A_{260}/ml (track 2); 5.0 A_{260}/ml (track 3); 3.75 A_{260}/ml (track 4); 2.5 A_{260}/ml (track 5); 1.25 A_{260}/ml (track 6). Designation by arrows as in Fig. 2.

reticulocyte) and animal microsomal membranes (dog pancreas) it was observed that the amount of added membranes affected the efficiency both of translation and of processing in an inverse manner. Similar results were obtained here with the plant-animal reconstitution system. It can be seen that increasing amounts of dog pancreas microsomal membranes added to the wheat germ cell-free system led to an increasing inhibition of mRNA translation (Fig. 2) but to a more efficient processing of the precursor of the IgG light chain (Fig. 3).

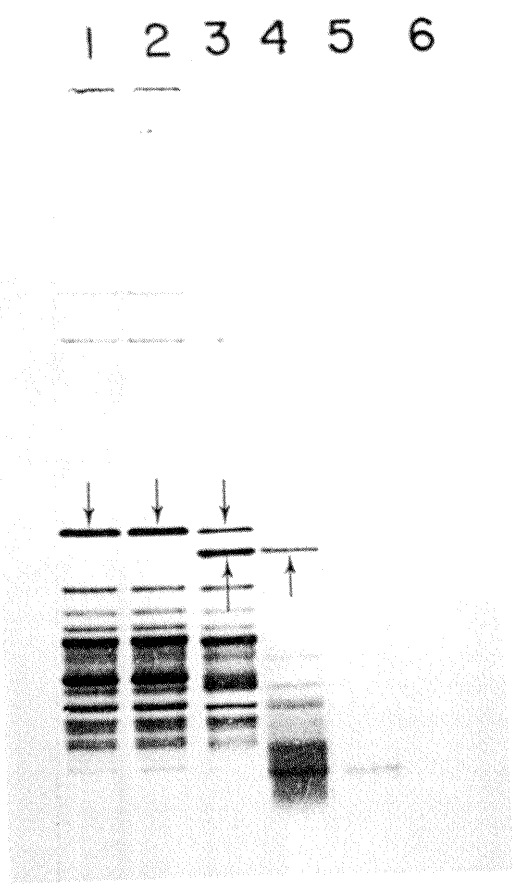

Fig. 4 Analysis by SDS-PAGE and AR of various co- and post-translational incubations with membranes, proteolytic enzymes and sodium deoxycholate. Track 1, control, i.e. post-translational incubation without any additions; track 2, post-translational incubation with membranes; track 3, co-translational incubation with membranes; track 4, co-translational incubation with membranes, followed by post-translational incubation with proteolytic enzymes; track 5, post-translational incubation with membranes, followed by incubation with proteolytic enzymes; track 6, as track 4 except that post-translational incubation with proteolytic enzymes was in the presence of 0.5% sodium deoxycholate. RM EDTA concentrations were 4.0 A_{260} units per ml. Designation by arrows as in Fig. 2.

Our data from previous reconstitution experiments (2) with animal components suggested that processing of the nascent presecretory protein is coupled to segregation, since only processed chains were found to be segregated, i.e. resistant to post-translational proteolysis. Moreover, it was shown (2) that processing and segregation are coupled and occur as co-translational,

not post-translational events. Similar results (Fig. 4) were obtained here with the plant-animal reconstitution system. It can be seen that processing took place only when dog pancreas microsomal membranes were present during translation (track 3), but not when they were present after translation (track 2). Furthermore, the co-translational presence of the microsomal membrane rendered the processed chains resistant to post-translational proteolysis (track 4), while under the same conditions the remaining unprocessed precursor chains were sensitive because they were presumably not segregated. Post-translational incubation with membranes not only failed to process the precursor (track 2); it also failed to segregate the precursor since it was not rendered resistant to subsequent proteolysis (track 5). That resistance to proteolysis is due to segregation rather than to an intrinsic property of the processed chain was shown by performing post-translational proteolysis in the presence of sodium deoxycholate in order to solubilize the protecting membranes. Under these conditions (track 6) the processed chain is sensitive to proteolysis.

DISCUSSION: The wheat germ cell-free system is widely used for the translation of mRNA's. It has a very low endogenous activity and it apparently does not contain membranes able to remove the signal sequence (1) of animal presecretory proteins. Our results show that supplementation of the wheat germ cell-free system with dog pancreas microsomal membranes results in removal of the signal sequence ("processing") from the nascent precursor of the IgG light chain and in intravesicular segregation of the processed chain. Processing was inferred from the observed reduction in size of the precursor to the size of the authentic light chain of IgG. It remains to be determined, however, whether processing was correct, i.e. whether the amino terminal amino acid sequence of the processed chain is identical to that of authentic IgG light chain. Segregation within microsomal vesicles of the processed light chain of IgG was inferred from its resistance to proteolytic enzymes which presumably cannot penetrate the vesicle lipid bilayer and therefore have no access to the

intraluminal space. That resistance to proteolysis of the processed chain is not one of its intrinsic properties but rather due to the membrane was shown by rendering the processed chain sensitive to proteolysis following solubilization of the membrane by sodium deoxycholate. Both processing and segregation were shown to be co-translational events. Post-translational addition of dog pancreas microsomal membranes to the wheat germ translation system resulted neither in processing nor in segregation of the precursor of the IgG light chain. These data strongly suggest that a functional junction has been established between a plant ribosome and an animal membrane during the translation of IgG light chain mRNA. However, analysis of this putative junction employing puromycin and high salt concentrations which have been used as probes for the characterization of the ribosome-membrane junction in native rough microsomes(8) remains to be performed in order to provide direct evidence that the reconstituted heterologous ribosome-membrane junction is equivalent to that in native rough microsomes.

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