Dithiothreitol and the Translocation of Preprolactin across Mammalian Endoplasmic Reticulum

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Abstract. The translocation mode of preprolactin (pPL) across mammalian endoplasmic reticulum was reinvestigated in light of recent findings that nascent secretory polypeptides synthesized in the presence of a highly reducing environment could be translocated posttranslationally and independently of their attachment to the ribosome (Maher, P. A., and S. J. Singer, 1986, *Proc. Natl. Acad. Sci. USA*, 83:9001–9005). The effects of the reducing agent dithiothreitol (DTT) on pPL synthesis and translocation were studied in this respect. The translocation of pPL was shown to take place only cotranslationally. The apparent posttranslational translocation was due to ongoing chain synthesis irrespective of the presence of high concentrations of DTT. When synthesis was completely blocked, no translocation was observed in the presence or absence of DTT. The synthesis of pPL was retarded by DTT, while its percent translocation was enhanced. The retardation in synthesis was reflected in reduced rates of initiation and elongation. As a consequence of this retardation, which increases the ratio of microsomes to nascent chains, and of possible effects on the conformation of nascent pPL and components of the translocation apparatus, DTT may expand the time and chain length windows for nascent chain translocation competence.

THE mechanism by which proteins are translocated across the endoplasmic reticulum (ER)[1] membrane or integrated in it is largely unknown. It was initially proposed that a nascent chain traverses the membrane vectorially while it is being synthesized, a process which became known as cotranslational translocation (Blobel and Dobberstein, 1975*b*; Palade, 1975; Sabatini et al., 1982). This mode of translocation seemed to apply for the mammalian ER. For membranes derived from many other sources, including bacteria (Date, et al., 1980; Müller and Blobel, 1984; Randall, 1983), glyoxysomes (Zimmerman and Neupert, 1980), chloroplasts (Highfield and Ellis, 1978; Cline, 1986), mitochondria (Schatz and Butow, 1983), and yeast (Rothblatt and Meyer, 1986; Waters and Blobel, 1986; Hansen, et al., 1986), posttranslational translocation of full-length proteins was demonstrated.

The question then arose of whether translocation across mammalian ER is only cotranslational or whether posttranslational translocation could also be demonstrated. It was found that preformed domains of a chimeric protein (a fusion between the lactamase signal sequence and the cytoplasmic protein globin; Perara et al., 1986), and the glucose transporter (an integral plasma membrane protein; Mueckler and Lodish, 1986), and full-length human preplacental lactogen, a mammalian secretory protein (Caulfield et al., 1986),

could be translocated across mammalian ER membranes posttranslationally while the nascent chains were still attached to the ribosome; releasing these chains from the ribosomes abolished the observed translocation. Two conclusions were made from these observations. First, preformed domains that could extend to full-length proteins remain translocation competent, and, second, the ribosome plays a role in the translocation process across ER membranes that is independent of its role in protein synthesis.

Although a role for the ribosome in the translocation process across ER membranes has been implicated for a long time (Borgese, et al., 1974; Blobel and Dobberstein, 1975*a*; Ibrahimi and Fuchs, 1987), direct evidence for this role or the nature of it has remained obscure. In a recent report (Maher and Singer, 1986), it was suggested that the role played by the ribosome in translocation could be bypassed by including a high concentration of the reducing agent dithiothreitol (DTT) during polypeptide synthesis. These results were interpreted by postulating that DTT, by preventing disulfide bond formation in the polypeptide being synthesized, maintains the chain in a translocation-competent state even after its release from the ribosome. This implied that the ribosome might play an indirect role in translocation by preventing the folding of the nascent polypeptide.

We have shown previously that nascent chains synthesized on *Escherichia coli* ribosomes were not translocated across mammalian ER (Ibrahimi and Fuchs, 1987). This evidence means that the ribosome plays a specific role in translocation that is not limited to the maintenance of the unfolded state

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1. *Abbreviations used in this paper*: ATA, aurintricarboxylic acid; ER, endoplasmic reticulum; PL, prolactin; pPL, preprolactin; RM-KN, salt-washed and nuclease-treated rough microsomes.

of the nascent chain but also involves specific recognition of this chain in the context of the ribosome. We have also shown that the signal sequence interacts functionally with the ribosome and that this interaction may play a role in targeting the nascent polypeptide to the membrane (Ibrahimi and Gentz, 1987). In this report it was shown that preprolactin (pPL) was translocated across mammalian ER membranes only co-translationally, irrespective of the DTT concentration during the translation process. However, in the presence of high concentrations of DTT, the rates of both initiation and elongation were retarded while percent translocation was enhanced.

Materials and Methods

Plasmid pSBP was provided by Dr. M. Wiedmann (Institute of Molecular Biology, Academic Sciences, 115 Berlin-Buch, German Democratic Republic). It contains a bovine pPL cDNA insert under the control of the SP6 promoter. SP6 RNA polymerase, ^{7}mGpppG, and proteinase K were from Boehringer, Mannheim, Federal Republic of Germany. Reticulocyte lysate (N150) and [^{35}S]methionine (1,000 Ci/mmol) were from Amersham International, Amersham, England. Phenylmethylsulfonyl fluoride (PMSF), ^{7}mGp, and aurintricarboxylic acid (ATA) were from Sigma Chemical Co., St. Louis, MO. Placental ribonuclease inhibitor was from Bethesda Research Laboratories, Inc., Gaithersburg, MD. Salt-washed and nuclease-treated microsomes (RM-KN) were prepared and used as previously described (Walter et al., 1981).

Protein Synthesis and Translocation Assays

Plasmid DNA was transcribed by purified SP6 polymerase in the presence of the capping structure ^{7}mGpppG as described (Krieg and Melton, 1984). The resulting mRNA was translated in reticulocyte lysate using the transcription reaction mixture without subsequent purification of the resulting mRNA. In a 25-μl translation assay, 1 μl of transcription mixture and 10 μl reticulocyte lysate were used under the conditions previously described (Bujard et al., 1987). [^{35}S]Methionine was used at 1 μCi/μl of translation mixture. For protein translocation, RM-KN was included in the translation mixtures at a concentration of 0.8A$_{280}$/ml (1 U) unless otherwise indicated. All incubations were done at 37°C for a total period of 80 min.

Co- and Posttranslational Treatments

Where indicated, DTT, ^{7}mGp, ATA, cycloheximide, or RM-KN were included in the translation reactions as described in the figure legends. The DTT concentrations do not include the 2.2 mM DTT that is normally present in all translation reactions. For the quantitation of the incorporation of [^{35}S]methionine in polypeptides, samples were spotted onto filter paper disks and dipped in ice-cold 10% TCA. The filters were boiled in 5% TCA, washed with ethanol/ether (1:1), ether, dried under a hot lamp and counted in liquid scintillation fluid. For protection assays, proteinase K was used at a concentration of 200 μg/ml in the presence of 10% sucrose. Digestion was allowed to proceed for 60 min on ice, after which it was stopped by the addition of PMSF to a final concentration of 2 mM.

Analysis of Translation Products

Samples were precipitated with 10% TCA on ice. The pellets were solubilized in sample buffer and subjected to SDS-PAGE on slab gels containing 10-15% acrylamide gradients as previously described (Laemmli, 1970). Radioactive protein bands were visualized by fluorography using EN^3-HANCE (New England Nuclear, Boston, MA) and quantitated by densitometry. Because pPL contains eight methionines, one of which is lost with the signal sequence upon cleavage to prolactin (PL), the following formulas were used to calculate percent processing and total synthesis.

$$\text{Percent processing} = \frac{8X}{8X + 7Y} \times 100,$$

where X and Y are densitometric counts in PL and pPL, respectively.

$$\text{Total synthesis} = \frac{8X}{7} + Y.$$
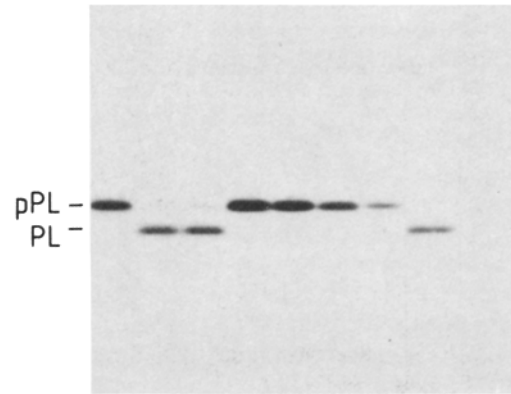
Figure 1. The translocation of pPL across mammalian endoplasmic reticulum occurs contranslationally. DNA of the plasmid pSBP was transcribed and the resulting mRNA was translated in reticulocyte lysates as described in Materials and Methods. Translations were supplemented with no components (lane *1*) or RM-KN, 1 and 2 U (lanes *2* and *3*). After 10 min of incubation at 37°C, ^{7}mGp, ATA, and cycloheximide were added to a final concentration of 4, 0.1, and 0.2 mM, respectively, to all incubations. This was followed by the addition of RM-KN after 10 min (lanes *4–7*). DTT was included at a final concentration of 25, 50, and 100 mM during translation (lanes *5–7*), respectively. Translations as in lane *2* were subjected to proteinase K treatment in the absence (lane *8*) and presence (lane *9*) of 0.5% Triton X-100. The results are shown after SDS-PAGE and autoradiography.

For determining percent synthesis that took place after the addition of microsomes, the counts in pPL from the start of incubation until the addition of microsomes were subtracted from the total synthesis counts at the end of the incubation period and the resulting value was expressed as percent of the total synthesis.

Results

I have investigated the mode of pPL translocation across the membrane of mammalian endoplasmic reticulum and the influence of DTT on its synthesis and translocation. Fig. 1 (lanes *2* and *3*) shows that pPL was efficiently processed to PL by RM-KN that were added at the start of the translation period. The processed PL was translocated into the microsomes as indicated by its resistance to digestion by proteinase K (Fig. 1, lanes *8* and *9*). When the microsomes were added after synthesis was stopped, no detectable level of processing was observed (Fig. 1, lane *4*). Under these conditions, the presence of 25, 50, or 100 mM DTT during chain synthesis also did not lead to a detectable level of processing (Fig. 1, lanes *5–7*).
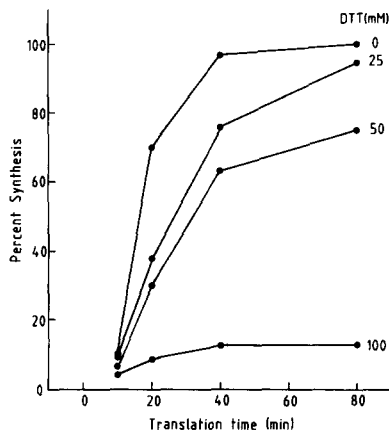
Effect of DTT on pPL Synthesis

Fig. 1 (lanes *4–7*) shows that DTT reduced the amount of pPL made in the translation assay. Therefore, I investigated the effect of DTT on pPL synthesis. It was found that DTT caused a concentration-dependent decrease in the rate of pPL chain synthesis. In the presence of 100 mM DTT, 87% inhibition of synthesis was observed after 80 min of incubation (Fig. 2). Because the translation system is completely
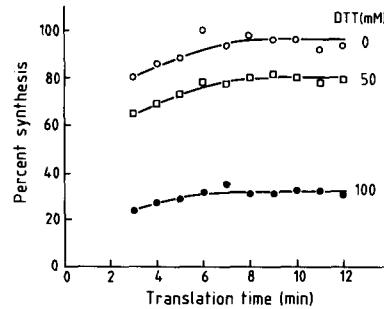
inactivated after 80 min of incubation, it was not possible to tell whether this inhibition was due to the inhibition of initiation, or elongation, or both. To discriminate between these possibilities, I determined the time course of [^{35}S]methionine incorporation into polypeptide chains in tightly synchronized translations in the absence and presence of DTT. In such a system, chain synthesis was completed during the early part of the incubation period before the translation system became inactive. Initiation was allowed to proceed for 2 min, followed by the addition of the initiation inhibitors, and the time course of [^{35}S]methionine incorporation thereafter was determined. Fig. 3 shows that incorporation ended in the first few minutes of further incubation. The level of synthesis in the presence of 50 and 100 mM DTT was 80 and 30% of that in the absence of DTT, respectively. These results led to the conclusion that initiation and/or early elongation in the presence of DTT were reduced.

A tightly synchronized translation system was also used to determine if elongation is affected by DTT. Initiation was allowed to proceed for 2 min in the absence of DTT. This was followed by the addition of the initiation inhibitors. Further elongation was measured in the absence and presence of DTT. Table I shows that the rate of elongation was reduced in the presence of DTT. The effect of DTT on chain synthesis was much more pronounced in the experiment presented in Fig. 3, suggesting that the retardation of chain synthesis by DTT is mainly at the level of initiation. It should be noted that in both Fig. 3 and Table I most of the radioactive counts accumulate during the first 2 min of incubation due to the fact that most of the methionines are in the first half of the pPL chain.

The effectiveness of the initiation inhibitor cocktail is shown in Fig. 4. It is clear that the cocktail inhibits initiation when added before the start of translation or 10 min after the start of translation.

Figure 3. DTT caused a decrease in polypeptide chain initiation. An incubation mixture containing all the components for the translation of pPL mRNA was prepared and divided into three equal portions on ice. To the first and second portions DTT was added to a final concentration of 50 and 100 mM, respectively; and to the third portion an equal volume of H$_2$O was added. After 2 min of incubation at 37°C, further chain initiation was inhibited by the addition of ^{7}mGp and ATA to a final concentration of 4 and 0.1 mM, respectively. At the indicated time intervals, equal samples were drawn out from each incubation mixture, spotted on filter paper disks and dipped in ice-cold 10% TCA. The disks were boiled in 5% TCA, washed with ethanol/ether (1:1), and ether, dried under a hot lamp, and counted in liquid scintillation fluid. The counts obtained in the presence and absence of DTT were calculated as a percentage of the highest value obtained and plotted versus time of sample withdrawal.

## Ongoing Chain Synthesis and the Apparent Posttranslational Processing of pPL

To demonstrate posttranslational translocation unequivocally, it is important to stop all protein synthesis before the addition of the membranes. Similarly, removal of the ribosomes before the addition of membranes is essential for studying the independence of the translocation process from the ribosome (Caulfield et al., 1986; Perara et al., 1986). Under conditions where this is not guaranteed, the observed translocation might be due to ongoing chain synthesis. Fig. 5 shows that this is indeed the case. After the addition of ^{7}mGp (2 mM final concentration), pPL synthesis continued at a significant but decreasing level for >20 min. The degree

Figure 2. DTT causes a concentration-dependent decrease in the rate of polypeptide chain synthesis. Transcription of plasmid DNA and translation of the resulting mRNA were done as described in Materials and Methods. Four incubations were done in the presence of 0, 25, 50, and 100 mM DTT. At the indicated time intervals, equal portions were drawn out from each incubation and the translation products were precipitated with TCA. The TCA pellets were solubilized in sample buffer and subjected to SDS-PAGE and autoradiography. The bands corresponding to pPL were quantitated by densitometry, calculated as a percentage of the highest value obtained, and plotted versus time of incubation.

Table I. Effect of DTT on Polypeptide Chain Elongation

Time	−DTT	+DTT
min	*cpm**	*cpm*
1	9,613	5,951
2	13,409	8,192
10	19,376	19,363
15	19,652	18,417

The translation of pPL mRNA was allowed to proceed for 2 min at 37°C followed by the addition of the initiation inhibitor cocktail (4 mM ^{7}mGp, 0.1 mM ATA). Immediately thereafter a sample was spotted on a filter paper disk and dipped in ice-cold 10% TCA; two samples were drawn out and added to either H$_2$O or DTT to give a 100 mM final concentration and the incubation was continued. From each of these two incubation mixtures, equal portions were drawn out at the time intervals indicated after the addition of the initiation inhibitor cocktail, spotted on filter paper disks, and dipped in ice-cold 10% TCA. The disks were boiled in 5% TCA, washed twice with ethanol/ether (1:1), twice with ether, dried under a hot lamp, and counted in liquid scintillation fluid. The counts obtained right after the addition of the initiation inhibitor cocktail (40,912) were subtracted from those obtained at each time interval. The resulting values are shown.
* The counts per minute incorporated as a result of chain elongation in the absence and presence of DTT are shown.

min
0   I - - - - -
10 - - - I I -
11 - - - - G G
    1 2 3 4 5 6

-pPL

-G
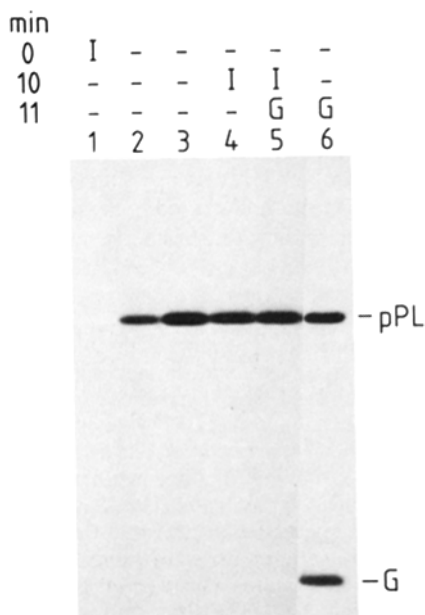
Figure 4. Inhibition of chain synthesis by ^{7}mGp (4 mM) and ATA (0.1 mM). Plasmid DNA was transcribed and the resulting mRNA was translated as described in Materials and Methods. Translations were supplemented with the initiation inhibitor cocktail (*I*) before the start of incubation (lane *1*), or 10 min after incubation (lanes *4* and *5*). This step was followed by the addition of globin (*G*) mRNA (lanes *5* and *6*). Lanes *2* and *3* were not supplemented with components. All translations were incubated for a total of 60 min at 37°C except for lane *2*, where translation was incubated for only 10 min, followed by TCA precipitation of the translation products. The results shown are after SDS-PAGE and autoradiography.

of percent processing correlated positively with that of ongoing translation in the presence of microsomes. The addition of cycloheximide (25 µM final concentration) together with the membranes resulted in 17, 7, and 0% synthesis at 5, 10, and 20 min.

DTT Retards the Rate of Synthesis of pPL and Enhances Its Percent Translocation across Mammalian ER Membranes

Because of the influence of DTT on translation and the positive correlation between ongoing pPL synthesis and translocation, I investigated the effect of DTT on pPL translocation. Fig. 6 shows that DTT enhanced the percent processing of pPL to PL. The processed PL was transported across the membrane as indicated by its resistance to proteinase K digestion (results not shown). The retardation of synthesis by DTT is also indicated as a curve plotted from Fig. 2.

Discussion

I have investigated the influence of DTT on the synthesis of pPL and its translocation across mammalian ER membranes. The results showed that pPL was translocated across ER membranes only cotranslationally. The apparent post-translational translocation was due to ongoing translation. DTT retarded pPL synthesis and enhanced its translocation efficiency.

These results are of direct relevance to two recent reports

in which posttranslational translocation across mammalian ER was reported. Full-length human preplacental lactogen was reported to be translocated posttranslationally, dependent on its attachment to the ribosome (Caulfield et al., 1986) or independent of this attachment if a high concentration of DTT was provided during the translation process (Maher and Singer, 1986). The results reported here with pPL indicated that when the membranes were added after chain synthesis had stopped completely, no translocation was observed in the absence or presence of DTT. Similar results were obtained previously with human IgG κ prelight chain in the absence of DTT (Blobel and Dobberstein, 1975b) and recently in the presence of high levels of DTT (D. Meyer, personal communication). However, it was observed that the amount of pPL synthesized in the presence of DTT was reduced. Using the exact conditions described before (Maher and Singer, 1986), i.e., allowing translation to proceed for 2 min in the presence of 0, 20, and 50 mM DTT, followed by the addition of ^{7}mGp (2 mM), which was intended to block further chain synthesis, and the addition of membranes at 1, 7, and 13 min for each DTT concentration, results that were in agreement with those reported here but only in part similar to those reported by Maher and Singer (1986) were obtained (results not shown). The main discrepancy is in the finding reported here that processing was observed in the absence as well as in the presence of DTT and that this processing decreased with the time of membrane addition. Maher and Singer (1986) reported no processing in the absence of DTT even when the microsomes were added 1 min after the addition of ^{7}mGP. At this time most of the chains that were initiated are still in their early phase of elongation and therefore, upon further incubation in the presence of microsomes, their elongation will be completed and they should be processed. Furthermore, from the results of Maher and Singer (1986), one can clearly see that DTT does stimulate the percent processing and retard chain synthesis in a concentration-dependent manner. Altogether, these results point to the possibility that
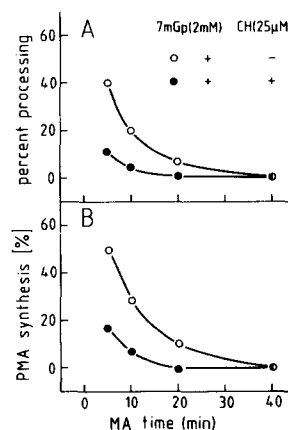
Figure 5. The apparent post-translational processing of pPL by RM-KN correlates positively with ongoing chain synthesis. Transcription and translation were done as described in Materials and Methods. A single incubation was started and ^{7}mGp (2 mM) was added 2 min later. Samples were drawn out at the indicated intervals and added to either TCA, RM-KN, or RM-KN plus cycloheximide (25 µM). The TCA-precipitated samples were kept on ice and the incubation of the other samples was continued for a total of 80 min; subsequently, the samples were precipitated with TCA. The pellets were solubilized and subjected to SDS-PAGE and autoradiography. The bands corresponding to PL and pPL were quantitated by densitometry. The percent processing and percent synthesis after the addition of microsomes were calculated as described in Materials and Methods. The resulting values were plotted versus the time of microsome addition. PMA, postmicrosomal addition; MA, microsomal addition.
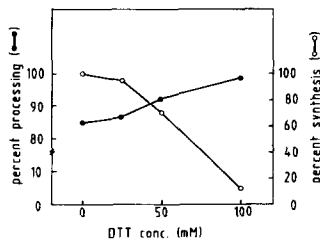
Figure 6. DTT enhances the percent processing of pPL and retards its rate of synthesis. The translation of pPL mRNA was allowed to take place in the presence of RM-KN and the indicated concentrations of DTT. Translation was done for 80 min at 37°. Translation products were subjected to SDS-PAGE and autoradiography. The bands corresponding to pPL and PL were quantitated by densitometry and the percent processing was calculated as described in Materials and Methods and plotted versus DTT concentration (*solid circles*). The values for percent synthesis of pPL (*open circles*) after 80 min of incubation at the indicated DTT concentrations are taken from Fig. 1.

the effect of DTT on translation and translocation might be related. These observations, together with the fact that chain synthesis continued for more than 20 min after the addition of ^{7}mGp (2 mM), led me to investigate whether the enhancement of percent translocation by DTT is due to its effect on the folding of completed and released pPL or whether this enhancement is exerted during chain synthesis. The results reported here provided evidence for the latter possibility.

The requirement of ongoing chain synthesis for translocation across ER membranes does not necessarily mean that the two processes are directly coupled. It was possible to uncouple the two processes before nascent polypeptide completion and release from the ribosome (Perara et al., 1986; Mückler and Lodish, 1986). I observed that nascent pPL became translocation incompetent when about half of the full-length polypeptide had been synthesized. Addition of the membranes past this chain length did not lead to a detectable level of translocation (unpublished results).

In a system where membranes are added before completion of the nascent polypeptide, further elongation would be necessary to complete the synthesis of these polypeptides, which will appear either as an untranslocated preprotein or translocated and processed protein. The translocation state of nascent polypeptides cannot be analyzed unless their synthesis is synchronized so that bands of discrete size can be detected. Such synchrony could be achieved by translating truncated mRNA, which results in a transient retention of the nascent chains on the ribosome (Ibrahimi et al., 1986; Haeuptle et al., 1986; Perara et al., 1986). Although preformed domains of proteins could be translocated posttranslationally, the question of whether these domains are translocated en bloc or threaded linearly remained open. In the latter case, a mechanism for maintaining the unfolded state of these domains or unfolding them would be necessary. DTT in high concentrations may provide such a mechanism in vitro, and, as a consequence, enhance the translocation process. The retardation of the rate of nascent polypeptide synthesis by DTT could also enhance the translocation process by increasing the ratio of microsomes to nascent pPL chains and by expanding the time window for nascent chain targeting to the membrane. Finally, the activity of components of the translocation apparatus itself could be influenced by DTT. The exact mechanism by which DTT retards nascent pPL synthesis and enhances its translocation remains a subject for further investigation.

It is possible that the ER has evolved a specialized mechanism for protein translocation. The ER occupies a unique position in the secretory pathway of a eukaryotic cell. Essentially all secretory and membrane proteins are made on ribosomes bound to the ER. The mechanism by which this association takes place and the role it plays in protein synthesis and translocation are not fully understood. The suggestion that this association is necessary to transfer the nascent polypeptide across the ER membrane cotranslationally served as a working hypothesis for more than a decade (Blobel and Dobberstein, 1975a, b; Palade, 1975; Sabatini et al., 1982). Posttranslational translocation was demonstrated for other types of membranes. It is conceivable that the cotranslational mode of translocation in the ER evolved with cellular compartmentalization as a means of ensuring that preproteins do not get mistargeted or even degraded if released into the cytoplasm. The posttranslational translocation of prepro-α-factor, unlike other proteins, across yeast and, to a much lesser extent, mammalian ER membranes, might be a reflection of the evolutionary history of the ER.

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