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Mechanisms of integration of *de novo*-synthesized polypeptides into membranes: Signal-recognition particle is required for integration into microsomal membranes of calcium ATPase and of lens MP26 but not of cytochrome b_5

(wheat germ cell-free translation system/salt-extracted microsomal membrane of dog pancreas/post-translational extraction of microsomal membrane at alkaline pH)

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ABSTRACT **We have investigated the *in vitro* integration into dog pancreas microsomal membranes of three integral membrane proteins that were synthesized *de novo* in a wheat germ cell-free translation system: calcium ATPase of rabbit sarcoplasmic reticulum, MP26 of bovine lens fiber plasma membrane, and rat liver cytochrome b_5. Biosynthetically these proteins show a common feature in that they are synthesized without a transient NH_2-terminal signal sequence. Two of these proteins, ATPase and MP26, were shown to require the recently discovered signal-recognition particle (SRP) [Walter, P. & Blobel, G. (1982) *Nature (London)* 299, 691–698] for integration. By this criterion, therefore, they each contain at least one uncleaved signal sequence. Surprisingly, however, the uncleaved signal sequence(s) of these two proteins did not induce the characteristic SRP-mediated translation arrest that was previously shown for a cleaved signal sequence. Unlike ATPase and MP26, cytochrome b_5 did not require SRP for integration into microsomal membrane. Thus, the distinction between an "insertion" sequence (specifying unassisted and opportunistic integration into any exposed membrane) and a "signal" sequence (directing integration into a specific membrane by a receptor-mediated mechanism) is a valid one. By assaying for SRP dependence, the two mechanisms of integration can now be experimentally distinguished.**

According to previous proposals (1), there are two distinct mechanisms for the integration of *de novo*-synthesized polypeptides into cell membranes. One is specified by an "insertion" sequence and proceeds unassisted into any exposed cell membrane, merely resulting in the anchorage of a hairpin-loop domain of the polypeptide chain into the lipid bilayer; such a hairpin loop could easily extend into the hydrophilic milieu on the other side of the membrane (2). The other one is mediated by a "signal" sequence and is dependent on a signal sequence-specific translocator that effects the translocation of a domain of the polypeptide from the biosynthetic compartment to the other side of a specific cell membrane; translocation was proposed to be interrupted by a "stop-transfer" sequence, thereby yielding precisely specified asymmetric integration of the polypeptide chain into the membrane (1).

Recently, advances have been made in the isolation and characterization of components of the translocator of one of the cell's translocation-competent membranes, the rough endoplasmic reticulum (RER). An 11S ribonucleoprotein consisting of six nonidentical polypeptides and one 7S RNA has been isolated (3, 4). Because the function of this particle is to decode the information in the RER-targeted signal sequence, it has been termed the signal-recognition particle (SRP) (4). SRP confers specificity on membrane integration (and translocation) by interacting with polysomes expressing RER-targeted signal sequences (5–7) and with a receptor that is an integral membrane protein in the RER membrane (8, 9).

For studies on the integration of polypeptides into the RER membrane, these advances made it possible to distinguish experimentally between signal sequence-mediated translocator-dependent integration on the one hand, and insertion sequence-mediated unassisted integration on the other hand. Thus, an *in vitro* translation system can be prepared that contains salt-extracted microsomal membranes free of the SRP (3). These membranes would be expected to remain competent acceptors for the integration of membrane proteins that use a SRP-independent insertion sequence. However, no integration into these membranes ought to occur for those *de novo*-synthesized membrane proteins containing a signal sequence. Integration of the latter would be expected to occur only in the presence of the SRP.

Using such SRP-depleted or -supplemented *in vitro* systems, we showed (10) that an integral membrane protein (δ subunit of the acetylcholine receptor) containing a large translocated domain and an NH_2-terminal cleaved signal sequence required SRP for integration into the microsomal membrane.

However, not all integral membrane proteins contain large translocated domains. Moreover, quite a few integral membrane proteins are *not* synthesized as larger precursors—i.e., they do not appear to contain a cleaved signal sequence. Are these polypeptides integrated into the RER membrane unassisted via an insertion sequence, or are they integrated in a SRP-dependent fashion via an *uncleaved* signal sequence? If the latter were the case, could the SRP be used to locate such an uncleaved signal sequence within these polypeptides by virtue of the ability of SRP to cause a site-specific elongation arrest (7), as it does with a cleaved RER-targeted signal sequence? To provide answers to these questions, we investigated the effects of SRP on cell-free synthesis and integration into heterologous microsomal membranes of three representative integral membrane polypeptides: calcium ATPase of rabbit muscle sarcoplasmic reticulum, MP26 of bovine lens fiber plasma membrane, and cytochrome b_5 of rat liver.

MATERIALS AND METHODS

Total RNA was extracted from the back muscle of 1-day-old rabbits or from rat liver by the $NaDodSO_4$/phenol/chloroform

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Abbreviations: K-RM, KCl-extracted microsomal membranes; SRP, signal-recognition particle; RER, rough endoplasmic reticulum.

method (11) and from adult bovine lenses by the $NaDodSO_4$/perchlorate method of Lizardi and Engleberg (12). In the latter method, carrier tRNA was added at the beginning of isolation to enhance the recovery of RNA.

The other procedures were as detailed elsewhere: translation of total RNA in the wheat germ cell-free system (13); preparation of KCl-extracted microsomal membranes (K-RM) and of SRP, both from dog pancreas (5); centrifugation of the translation products in sucrose step gradients under alkaline conditions to separate *de novo*-synthesized integral membrane polypeptides that were integrated into microsomal membranes from those that were not integrated (14); immunoprecipitation of $NaDodSO_4$-denatured translation products (11) with monospecific antibodies (see below); $NaDodSO_4$/polyacrylamide gel electrophoresis of the immunoprecipitates (11, 15); and fluorography (16).

[^{35}S]Methionine (1,000 Ci/mmol; 1 Ci = 37 GBq) was obtained from New England Nuclear.

Immunological Reagents. Our anti-MP26 antiserum had been prepared using as antigen a polypeptide band eluted from the MP26 region of preparative $NaDodSO_4$/polyacrylamide gels of isolated bovine lens fiber membranes (17). For immunoselection of the crude antiserum, similar preparations of MP26 were further purified by hydroxylapatite chromatography in $NaDodSO_4$ (18) (data not shown). Material from a narrow "cut" of the elution peak was coupled to CNBr-activated Sepharose C1-4B to serve as an affinity matrix.

In the case of cytochrome b_5, the original antiserum had been raised against the water-soluble tryptic fragment of this molecule. For immunoselection we used intact cytochrome b_5 that had been isolated by an entirely different procedure (kindly provided by K. Mihara, Osaka University, Japan) and further purified this material in denatured form by preparative $NaDodSO_4$/polyacrylamide gel electrophoresis. These procedures eliminated the possibility that trace contaminants present in the immunogen preparation would also be present on the affinity matrix.

The specificity of a sheep antiserum to calcium ATPase of rabbit sarcoplasmic reticulum (a generous gift of Paul DeFoor and Sidney Fleischer) has been established (14).

RESULTS

Total RNA was translated in the wheat germ cell-free system containing [^{35}S]methionine. This system was supplemented, cotranslationally or post-translationally, either with SRP or with K-RM (SRP-free) of dog pancreas or with both. The translation mixture was then centrifuged under alkaline conditions, yielding a supernatant fraction containing nonintegrated polypeptides and a pellet fraction containing microsomal membranes with newly integrated polypeptides. After solubilization with $NaDodSO_4$, the polypeptides of interest in these fractions were immunoprecipitated, subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis and visualized by fluorography. For quantitation, bands were excised and the radioactivity was determined by liquid scintillation counting. Thus, membrane-integrated polypeptides are operationally defined here as those that resisted extraction from the microsomal membrane under alkaline conditions (19).

Potentially, an uncleaved signal sequence may be detectable by these assays in several ways. First, only in the presence of SRP (and in the absence of K-RM) would one expect a signal sequence-induced elongation arrest (7). As the arrest should be site specific (7), a discrete peptide should be detectable (after hydrolysis of the peptidyl tRNA bond). From the size of the arrested peptide one should be able to estimate the location of the signal sequence. Second, the signal sequence-mediated elongation arrest should be abolished after the addition of K-RM, with the concomitant conversion of the arrested peptide to the full-length product (7). And third, integration into K-RM should occur in the presence of SRP but not in its absence (10).

The data will be presented separately for each of the three integral membrane proteins preceded, in each case, by a brief description of what is currently known about their asymmetric topology in the membrane and their biosynthesis.

Sarcoplasmic Reticulum Calcium ATPase. The enzyme of rabbit skeletal muscle is a polypeptide of an estimated mass of 119,000 daltons (20). The precise topology of the polypeptide chain with respect to the lipid bilayer and its hydrophilic environment on either side is unknown. Untranslocated domains exposed to the cytoplasm appear to alternate with at least three membrane-embedded loops (21). Some of these loops may contain domains exposed to the intracisternal milieu. The NH_2 terminus is known to be part of one of the untranslocated domains exposed to the cytoplasm (22). In cell-free translation experiments, the enzyme was shown to be synthesized without a transient NH_2-terminal signal sequence (14). Yet, for integration, the cotranslational presence of microsomal membranes was required; however, only unextracted (i.e., SRP-containing) microsomal membranes have previously been tested (14). The requirement for the cotranslational presence of microsomal membranes could be interpreted in two ways. Integration could proceed in a SRP-dependent fashion, mediated by an uncleaved signal sequence. Alternatively, and equally likely, integration could be SRP independent and mediated by an insertion sequence, especially if such a sequence could be expressed only by a nascent and not by a completed chain.

The data in Fig. 1 show that calcium ATPase requires the SRP for integration into microsomal membranes and, by this criterion, strongly suggest that this polypeptide contains an uncleaved signal sequence. Thus, when SRP was absent (*A*), only a small fraction of the total *de novo*-synthesized calcium ATPase (lane T) associated with the microsomal membrane (lane P), whereas the bulk remained in the supernatant (lane S). As SRP was added in increasing amounts (*B* and *C*), more and eventually most of the total *de novo*-synthesized calcium ATPase was integrated into the microsomal membranes (lanes P).

Curiously, however, the uncleaved signal sequence of calcium ATPase did not induce a SRP-mediated elongation arrest, at least not a detectable one. Although there was ≈20% inhibition of calcium ATPase synthesis in the presence of the highest concentration of SRP (compare lanes T of *D* and *E*), this inhibition was apparently nonspecific and not due to elongation arrest because it was not abolished by K-RM (compare lanes T of *E* and *C*). In fact, the nonspecific inhibition caused by K-RM and that caused by SRP (compare lanes T of *A*, *E*, and *C*) were additive.

The data in Fig. 1 also show that both the recognition of the uncleaved signal sequence by SRP and the subsequent integration into K-RM are strictly translation-coupled events. Thus, integration was not observed when SRP was present cotranslationally and membranes were added post-translationally (*E*, lane P) or vice-versa, when membranes were present cotranslationally and SRP was added post-translationally (*A*, lane P).

MP26 of Lens Fiber Plasma Membrane. MP26 is the major polypeptide of bovine lens fiber plasma membranes, with a mass of 26,000 daltons. The topology of this integral membrane protein is unknown. In particular, it is not known whether it possesses translocated hydrophilic domains. There is some evidence suggesting that the NH_2 terminus is part of an untranslocated domain (23, 24). Cell-free translation of mRNA showed that MP26 is not synthesized as a larger precursor and

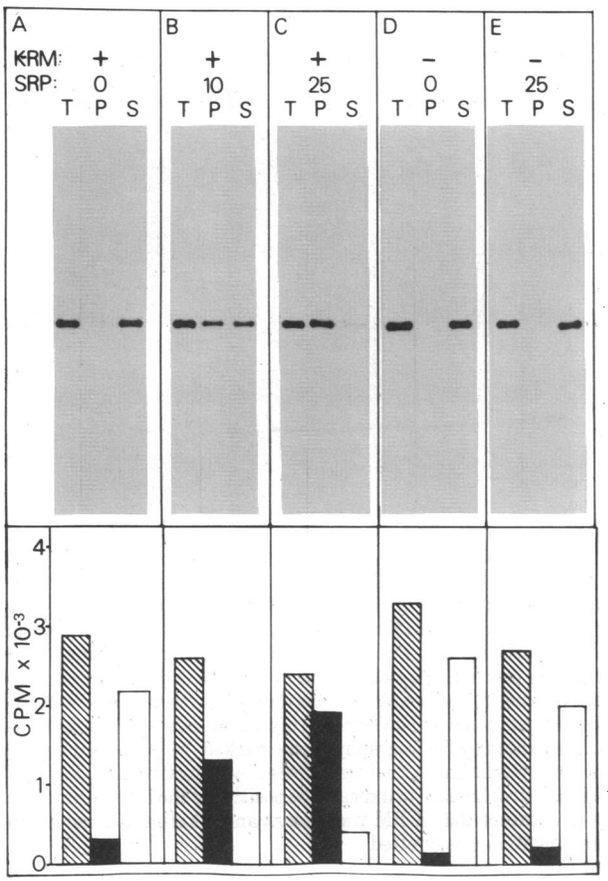

FIG. 1. SRP-dependent integration of *de novo*-synthesized calcium ATPase into microsomal membranes. Five 160-μl translation reactions were set up containing total RNA from rabbit muscle. SRP and K-RM were either absent or present at the onset of translation: K-RM at a concentration of 19 equiv per 50 μl and SRP at a concentration of 10 or 25 equiv per 50 μl. Translations were carried out for 2 hr at 22°C, at which point no further incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable material was detected. At this time, the SRP or K-RM (or both) was added to the reaction so that the ultimate concentrations of SRP and K-RM were identical in all five reaction mixtures. A post-translational incubation at 22°C for a further 2 hr was then carried out. Each reaction mixture was then divided in half. One half was denatured with $NaDodSO_4$ and immunoprecipitated with anti-ATPase serum (lanes T). The other half was adjusted with 1.0 M NaOH to pH ≈11.5. This mixture was then separated on alkaline sucrose step gradients into pellet and supernatant fractions as described (14). ATPase was immunoprecipitated from the pellet (lanes P) and supernatant (lanes S) separately. The immunoprecipitates were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis and radioactive bands were visualized by fluorography. Quantitation was carried out by excising the ATPase bands and determining the radioactivity by liquid scintillation counting. Note that in each case the sum of the radioactivity in the pellet and supernatant fractions is slightly less than in the unfractionated material. This is probably due to losses of material during fractionation on the sucrose gradient.

that it requires the cotranslational presence of microsomal membrane (K-RM has not been tested) for integration (23). Thus, as is the case for calcium ATPase, one could conceive of a signal sequence-mediated or an insertion sequence-mediated mechanism of integration.

The data obtained for MP26 are analogous to those obtained for calcium ATPase (Figs. 2 and 3). Thus, integration of MP26 required SRP (Fig. 2). MP26, therefore, is likely to contain an uncleaved signal sequence. Again, the uncleaved signal sequence failed to induce a detectable SRP-mediated elongation arrest, even at very high SRP concentrations (see Fig. 3A). The

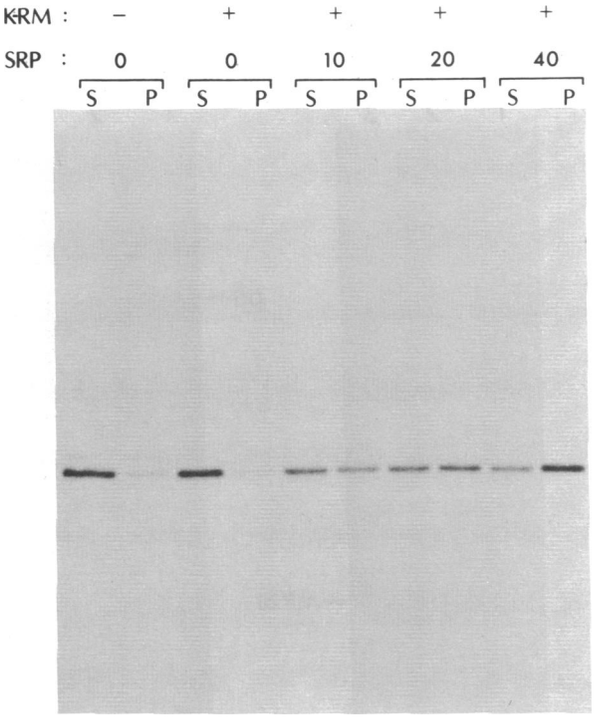

FIG. 2. SRP-dependent integration of *de novo*-synthesized lens MP26 into microsomal membranes. Five 100-μl translation reactions were set up containing total RNA from bovine lens. K-RM and SRP were either absent or present at the onset of translation: K-RM at a concentration of 19 equiv per 50 μl and SRP at concentrations of 10, 20, or 40 equiv per 50 μl. Translations were carried out for 1 hr at 28°C. Each reaction mixture was then fractionated on alkaline sucrose step gradients (see Fig. 1) into a supernatant and a pellet fraction. Lens MP26 was precipitated with affinity-purified antibodies, subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis, and visualized by fluorography.

data of a control experiment showing SRP-mediated elongation arrest for the δ subunit of the acetylcholine receptor (10) are shown in Fig. 3*B*.

Cytochrome b_5. Cytochrome b_5 is an integral membrane protein located in several cell membranes. It consists of a large untranslocated domain (≈11,000 daltons) containing the NH_2 terminus and a hydrophobic COOH-terminal segment (≈5,000 daltons) interacting with the hydrophobic core of the lipid bilayer. It is still not clear whether this interaction is in the form of a loop, with a few COOH-terminal residues exposed in the cytoplasm, or in the form of a transmembrane segment, with the COOH-terminal residues translocated (25–28). Cell-free translation of mRNA has shown that the protein is *not* made as a larger precursor (29). However, unlike calcium ATPase and MP26, newly synthesized cytochrome b_5 can be post-translationally integrated into microsomal membranes (30, 31). Detergent-extracted and purified cytochrome b_5 can be integrated into lipid vesicles (25–28, 31). Taken together, these data suggested that the integration of cytochrome b_5 is not mediated by a signal sequence but by an insertion sequence and, therefore, is independent of SRP. The data in Fig. 4*A* show that integration of *de novo*-synthesized cytochrome b_5 into microsomal membranes, in fact, does not require SRP and, therefore, is not mediated by a signal sequence. Moreover, SRP did not affect chain elongation (Fig. 4*B*).

DISCUSSION

Our data show that the recently discovered SRP can be used to determine whether a signal sequence is present in those in-

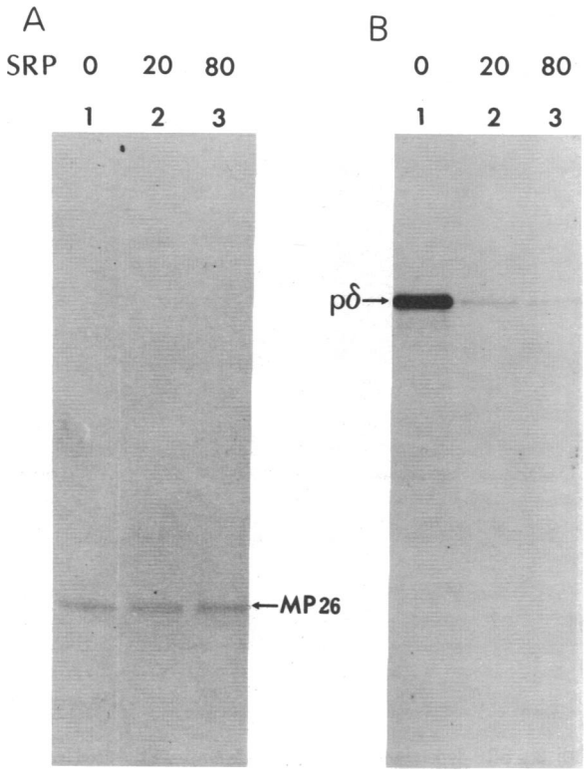

FIG. 3. SRP does not arrest the synthesis of MP26. Translation reactions lacking K-RM were programmed with either calf lens RNA (*A*) or *Torpedo californica* RNA (*B*). SRP was excluded from the reaction (lanes 1) or included at 20 (lanes 2) and 80 (lanes 3) units per 50 μl of reaction mixture. Samples were subsequently immunoprecipitated with affinity-purified anti-MP26 antibody (*A*), or antibody to the δ subunit of acetylcholine receptor (*B*) (10). pδ to the left of lane 1 in *B* indicates pre-δ subunit of acetylcholine receptor.

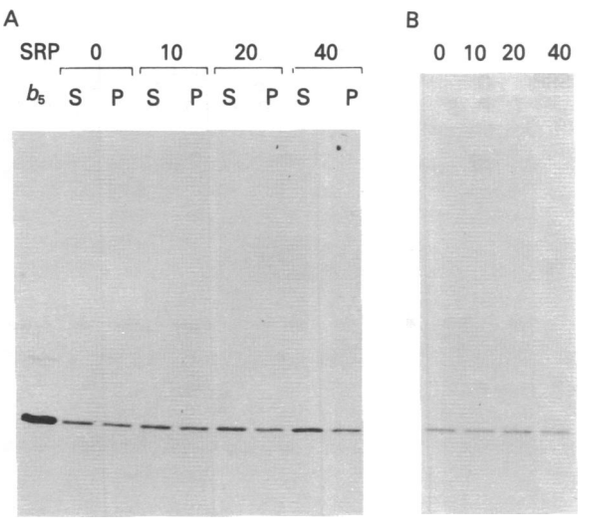

FIG. 4. SRP affects neither integration nor synthesis of cytochrome b_5. (*A*) Five 100-μl translation mixtures were set up containing total rat liver RNA, 40 equiv of K-RM and either no SRP or SRP in the concentrations indicated (equiv per 50 μl of translation mixture). After translation for 1 hr at 28°C and fractionation in alkaline sucrose gradients (see Fig. 1), cytochrome b_5 was precipitated in the supernatant (lanes S) and pellet fractions (lanes P) with affinity-purified antibodies, subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis, and visualized by fluorography. Lane marked "b_5" shows an aliquot of purified cytochrome b_5 that was labeled by reductive methylation (32) and coelectrophoresed to indicate the position of the mature protein. (*B*) As in *A* except that K-RM was absent and alkaline sucrose gradient centrifugation was omitted.

tegral membrane proteins in which the existence of such a sequence is otherwise not apparent. We have analyzed three representative examples: calcium ATPase of rabbit sarcoplasmic reticulum, MP26 of bovine lens fiber plasma membrane, and cytochrome b_5 of rat liver. All three of these polypeptides were known to be synthesized without a cleaved signal sequence—i.e., there is no proteolytic processing of an NH_2-terminal peptide on integration into microsomal membranes. Moreover, none of these three polypeptides has large translocated domains; thus, *a priori*, there is no conceivable need for the existence of an uncleaved signal sequence in these polypeptides; integration could instead occur entirely unassisted, only directed by insertion sequences (see Introduction and ref. 1).

Our *in vitro* integration system consisting of a wheat germ cell-free translation system, SRP, and salt-extracted SRP-depleted microsomal membranes showed that SRP was required for the integration into microsomal membrane of ATPase and MP26, but not of cytochrome b_5. These data strongly suggest that the ATPase as well as MP26 each has at least one uncleaved signal sequence, whereas cytochrome b_5 does not and is integrated instead via an insertion sequence. Thus, the previously made theoretical distinction between a signal sequence and an insertion sequence is a valid one. These two topogenic sequences (1) can now be experimentally distinguished by testing for SRP dependency of integration.

Curiously, the uncleaved signal sequences of ATPase and MP26 did not induce a SRP-mediated elongation arrest such as that previously observed for several NH_2-terminal cleaved signal sequences. Thus, our hope to deduce the location of the uncleaved signal sequence from the size of the arrested nascent chain (see ref. 7) could not be realized. We cannot, however, exclude the possibility that a translation arrest was in fact induced also by the uncleaved signal sequences, but that it was much less stable and, therefore, affected only the rate of synthesis and not the yield of completed chains.

Although our data strongly suggest that integration took place, they do not permit any conclusions as to the fidelity of integration. Both the ATPase and MP26 apparently contain several membrane-traversing loops and no large translocated domains. Therefore, the presence of a signal sequence in these two polypeptides does not appear to result in the translocation of a large domain. The translocation process instead appears to be rapidly aborted. It was previously proposed (1) that the interruption of the translocation process is achieved by another distinct segment of the polypeptide chain, termed the "stop transfer" sequence. Because the NH_2 termini of ATPase and MP26 are known to be untranslocated (i.e., exposed in the cytoplasm), an uncleaved signal sequence and a stop-transfer sequence could at best be expected to cause the integration of only *one* hairpin loop with its apex translocated to the other side of the membrane and its two termini untranslocated. Further integration of the COOH-terminal bulk portion of the chain could theoretically continue by a program of alternating-signal and stop-transfer sequences so as to stitch the remainder of the polypeptide into the membrane in the form of several loops (1). Alternatively, integration could continue instead by a program of insertion sequences, each loop being specified by one insertion sequence (1). If this were the case, the raison d'etre of a *single* signal sequence in ATPase and MP26 is not to translocate a large domain, but is to ensure that the site of integration is limited to the RER.

Noteworthy is our finding that integration is strictly dependent on the cotranslational presence of both SRP and micro-

somal membranes. Thus, the chains of ATPase and MP26, although synthesized in the presence of SRP, and therefore given a chance to interact with it, cannot be post-translationally integrated into the microsomal membranes. Thus, recognition by the SRP and subsequent integration are coupled events. Moreover, the finding that there was no integration into cotranslationally added microsomal membranes when SRP was absent suggested that putative insertion sequences cannot be expressed, either co- or post-translationally, unless the signal sequence first initiates the integration process cotranslationally. This again would constitute a safety mechanism against opportunistic integration into other exposed cell membranes and would ensure that the port of membrane entry is limited to the RER with sorting to other membranes occurring subsequently.

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