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Uncoupling of Translocation Across Microsomal Membranes From Biosynthesis of Influenza Virus Hemagglutinin

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This communication presents our recent studies on the biosynthesis of influenza virus hemagglutinin (HA) in a mammalian-cell-free system and its translocation across microsomal membranes. RNAs coding for wild-type (full-length) and mutant (truncated) forms of HA were generated by in vitro transcription by using bacteriophage T7 DNA-dependent RNA polymerase. These RNAs were translated in a rabbit reticulocyte system that was supplemented with dog pancreas membranes, either before translation was initiated or after it had been artificially terminated with the antibiotic cycloheximide. All forms of HA could be cotranslationally translocated. However, only truncated molecules (83% of full length) could translocate after protein synthesis had been terminated. Posttranslational translocation was dependent on the presence of a functional N-terminal signal sequence and occurred only in the presence of ribosomes. The molecular mechanism of protein targeting and translocation across the membrane of the endoplasmic reticulum is discussed based on the signal hypothesis.

Key words: *in vitro* translation, endoplasmic reticulum, influenza hemagglutinin

For several years, it was believed that translocation of mammalian proteins across the membrane of the rough endoplasmic reticulum (ER) is strictly coupled to their biosynthesis (see [1–4] for reviews). Several components have been identified and characterized that played important roles in this process (eg, signal recognition particle [SRP] [5,6] and SRP receptor, also termed docking protein [7–10]). Using cell-free protein-synthesizing systems derived from wheat germ, it has been shown that SRP specifically arrests the biosynthesis of nascent secretory and transmembrane proteins, presumably by interacting with their amino-terminal hydrophobic signal peptides. Translation resumes after interaction between the ribosome-bound SRP and

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the SRP receptor, an integral membrane protein of the endoplasmic reticulum. The nascent proteins are then transferred across the membrane to the lumenal side of the ER by a process whose molecular mechanisms are not yet understood.

Recent evidence, however, indicates that SRP-mediated translational arrest is less than complete in cell-free systems derived from reticulocyte lysates [11,12]; in some cases, it amounts to no more than a transient delay in biosynthesis of nascent secretory proteins [12–14]. Furthermore, in systems derived entirely from mammalian sources, translocation is not obligatorily cotranslational and can occur after biosynthesis of a protein is complete (see [2–4] for reviews). The list of proteins that can be translocated posttranslationally is growing and includes the human erythrocyte glucose transporter [15], human placental lactogen [16], honeybee prepromelittin [17], and a fusion protein consisting of the signal sequences of β-lactamase and human α-globin [18]. In all these cases, posttranslation translocation is both ATP- and ribosome-dependent. A yeast secretory protein, the precursor to α-factor, can also be posttranslationally translocated in a yeast-cell-free translation system [19–21]. The process is ATP-dependent but, by contrast to the situation in mammalian systems, it occurs in the absence of ribosomes.

To define more precisely the requirements for translocation of proteins in a mammalian-cell-free system, we have analyzed the ability of a well-characterized transmembrane protein, influenza virus hemagglutinin (HA), to be co- and posttranslationally translocated across microsomal membranes. We show that full-length HA can be translocated co- but not posttranslationally. By contrast, truncated forms of the molecule, less than 83% of the wild-type molecule in length, can be translocated across microsomal membranes after their synthesis is completed. This posttranslational translocation, which is comparatively inefficient, requires the presence of ribosomes. A possible pathway for protein translocation, based on these results, is discussed.

MATERIALS AND METHODS

Construction and Mutagenesis of a Vector for Transcription of a cDNA Encoding Influenza Hemagglutinin

Cloned cDNA encoding the HA of the A/Japan/305/57 strain of influenza virus [22] was inserted into an expression vector, pT7/T3-19 (BRL), by using standard methods [23]. To construct a mutation in the N-terminal hydrophobic signal of HA, wild-type HA cDNA was cloned into M13mp18 by using HindIII and BamHI restriction sites. Site-directed mutagenesis of HA cDNA with mismatched oligonucleotides and identification of the desired mutants was carried out as described by Zoller and Smith [24]. Mutant forms of HA cDNA were then subcloned into pT7/T3-19 for in vitro expression. A restriction map of the recombinant vector, designated pT7HA-Jap, and the encoded HA polypeptide are schematically represented in Figure 1.

Translation in Mammalian Cell-Free Systems of Transcripts Generated In Vitro

Transcription of wild-type and mutant forms of HA cDNA in pT7/T3-19 was carried out by using bacteriophage T7 DNA-dependent RNA polymerase according to the manufacturers' instructions (BRL). mRNA was synthesized from 0.5 μg of linearized DNA plasmid and aliquots of the transcription reaction mixture were used

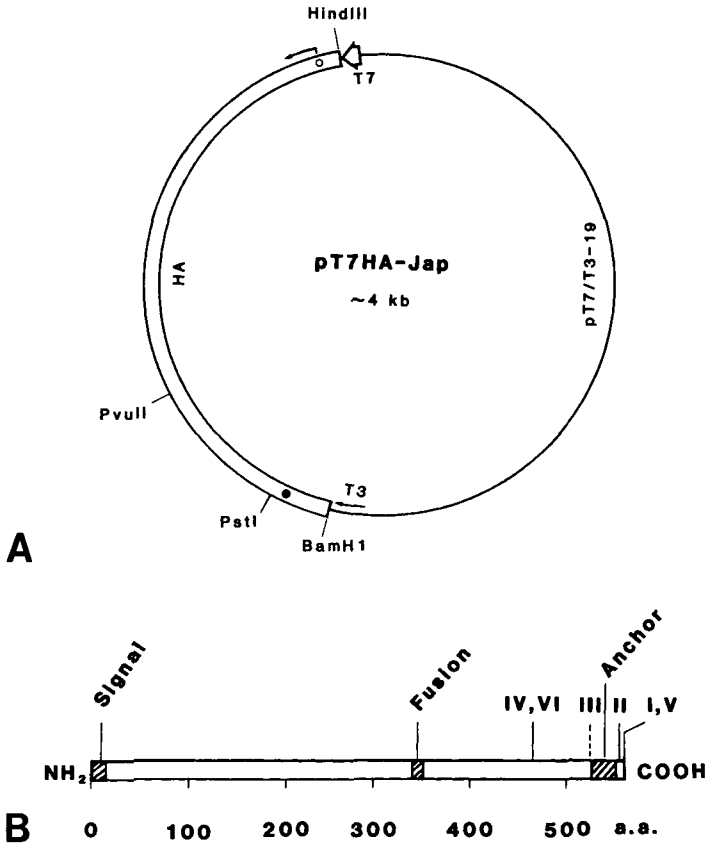

Fig. 1. Restriction map of pT7HA-Jap and schematic representation of HA peptide. **A:** Restriction map of pT7HA-Jap. Only the restriction sites used to linearize HA cDNA are indicated. **B:** Schematic representation of HA polypeptide. Three hydrophobic domains of HA are hatched with their respective functions indicated. Roman numerals represent the full-length (I,V) and truncated forms (II, III, IV, VI) marked with arrows at the end of each molecule. The length scale (amino acids) is also shown below the polypeptide. Restriction sites used to linearize HA cDNA for each polypeptide are: BamH1 for I and V; PstI for II; BamH1 for III (the cDNA for this particular case was from independent construct); PvuII for IV and VI.

directly in a cell-free translation system derived from rabbit reticulocytes (Promega Biotech). Each translation reaction contained approximately 0.5 μg of mRNA and was incubated at 30°C for 40 min. Some translation reactions were supplemented with rough microsomes from dog pancreas (a gift from Peter Walter, UCSF) either before or after termination of protein synthesis (see below).

Posttranslational Treatment and Translocation Assays

Cycloheximide (Sigma) was added at the end of translation to a final concentration of 0.1 mg/ml. After storage for 20 min on ice, rough microsomes were then added to some samples and the incubation was continued for a further 40 min. Translocation of HA across microsomal membranes was detected by (1) a decrease in the electrophoretic mobility of the molecule caused by attachment of mannose-rich oligosaccharides and (2) protease-protection assays. In the latter case, duplicate

samples were either treated with Triton-X 100 or not treated, and then were digested with proteinase K (0.05 mg/ml) for 20 min on ice. Phenylmethylsulfonyl fluoride (PMSF) (Sigma) was then added to a final concentration of 0.5 mg/ml and the samples were then stored for 15 min on ice. Radiolabeled HA was immunoprecipitated with a polyclonal antiserum that recognizes both native and denatured forms of the protein and then analyzed by SDS–polyacrylamide gel electrophoresis as described previously [25,26]. [^{35}S]methionine-labeled bands were visualized by fluorography.

RESULTS AND DISCUSSION

We have analyzed the translocation of wild-type, truncated, and mutant forms of influenza virus HA into microsomes both during and after completion of biosynthesis in a mammalian-cell-free protein-synthesizing system. Results are summarized in Table I. Typical results are shown in Figure 2. Full-length HA carrying a wild-type signal (panel I) is translocated when rough microsomes are present during biosynthesis of the molecule. The translocated molecules become glycosylated (as judged by a decrease in electrophoretic mobility, lane 2) and resistant to exogenously added protease (lane 5). As expected, disruption of microsomes with Triton-X 100 renders the translocated HA sensitive to protease. Because no translocated HA is detected when microsomes are added to the reaction after synthesis of radiolabeled HA has been terminated by the addition of cycloheximide (lane 4), we conclude that translocation of full-length HA is obligatorily cotranslational in this cell-free system. Cotranslational translocation is dependent on the presence of a functional N-terminal signal sequence, since full-length HA carrying arginine residues in place of the leucines at positions 6 and 8 was not detectably translocated, even when microsomes were present during biosynthesis (panel V, lanes 8 and 9).

By contrast, a truncated form of HA (83% of full-length) was translocated both co- and posttranslationally (panel IV). Although the efficiency varied from experiment to experiment, cotranslational translocation (lane 2) was always much more efficient than posttranslational translocation (lane 3). In the experiment shown in Figure 2, more than 50% of the truncated HA is translocated when microsomes are present during the entire period of incubation; however, less than 5% of HA is translocated when microsomes are added to the system after synthesis of HA has been terminated with cycloheximide. Nevertheless, the small fraction of HA that is translocated into microsomes posttranslationally is both glycosylated and resistant to protease (lanes 5

TABLE I. Summary of the Translocation of Wild-Type and Mutated Forms of HA Across Microsomes

HA construct	Signal	Length, aa[a] %	Translocation Co	Translocation Post
I	WT	562 (100)	+	–
II	WT	555 (99)	+	–
III	WT	536 (95)	+	–
IV	WT	465 (83)	+	+
V	MUT	562 (100)	–	–
VI	MUT	465 (83)	–	–

[a]The number in parentheses represents a relative size of the truncated form to the full-length molecule, which is 562 amino acids (aa) in length.

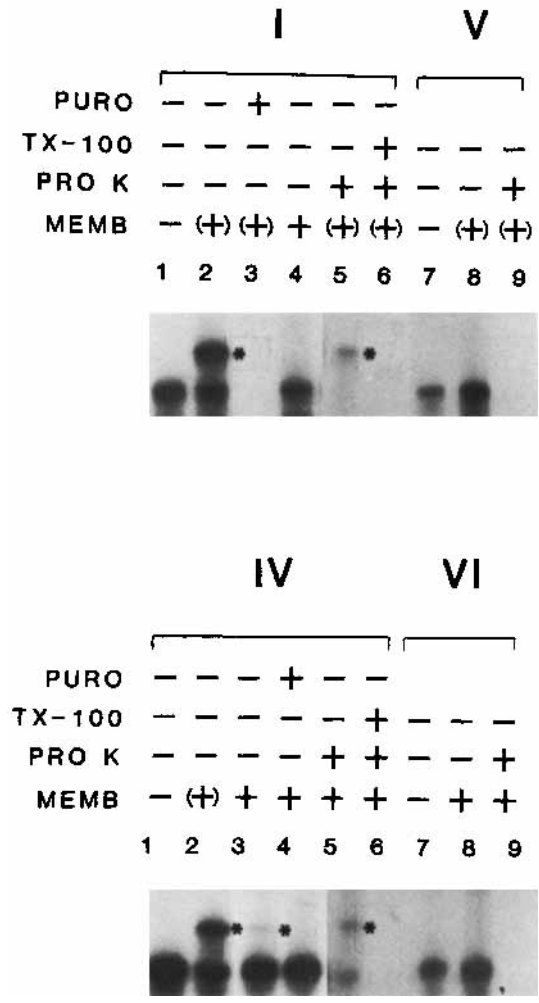

Fig. 2. In vitro expression of HA cDNA. In vitro translation products were immunoprecipitated and analyzed by SDS-PAGE and fluorography. **Upper panel:** Full-length HA, I and V, with wild-type **(lanes 1–6)** or mutated **(lanes 7–9)** signals synthesized either with or without microsomes. Addition of microsomes after translation is marked with "+" symbol; otherwise addition before translation with symbol in parentheses. Lane 3, puromycin and microsomes were included in the reaction mixture before protein synthesis. After translation, the proteins were subjected to the proteinase K treatment in the presence (lane 6) or absence (lanes 5 and 9) of 0.5% Triton X-100. The translocated forms are marked with "*". **Lower panel:** Truncated HA, IV and VI, with wild-type **(lanes 1–6)** or mutated **(lanes 7–9)** signals synthesized in the conditions indicated. After synthesis, proteins were treated as described for the upper panel except that puromycin (lane 4) was added after protein synthesis. Lane 5 was overexposed to visualize a low level of the translocated band (compare lanes 3 and 5). The symbols are the same as for the upper panel.

and 6). Posttranslational translocation of truncated HA was abrogated both by puromycin (lane 4) and by the presence of a mutated signal sequence (compare lanes 3 and 8). We therefore conclude that this process depends on the integrity of the ribosomal-mRNA complex and that it uses the same hydrophobic signal that is responsible for cotranslational translocation.

These results raise several questions. Why should posttranslational translocation be restricted to molecules of HA that are less than full length? Why should the process require a functional signal sequence and integrity of the ribosome-mRNA-nascent protein complex? It seems likely to us that the failure of full-length HA to translocate is a consequence of the proteins' folding into a conformation that is incompatible with its transfer across the microsomal membrane. This hypothesis has been proposed previously by Maher and Singer [27], who suggest that the formation of disulphide bonds in proteins synthesized in cell-free systems is sufficient to prevent their posttranslational translocation. This explanation cannot explain our observations, since the inclusion of reducing agents in the in vitro system did not allow full-length HA to be posttranslationally translocated (unpublished results). Even in the absence of disulfide bond formation, it therefore seems that HA can fold into configuration(s) that are unacceptable for translocation. The longer the polypeptide, the greater are the chances that it will enter such an unacceptable configuration. Full-length HA seems to enter such unacceptable configurations rapidly. However, a truncated form of HA, lacking 97 amino acids from the carboxy terminus, displays some ability to be posttranslationally translocated. Presumably the low efficiency of this process reflects the small proportion of the truncated molecules that have avoided forming structures that cannot be translocated.

The mRNAs that encode truncated forms of HA (Table I) were generated by in vitro transcription of linearized plasmids and therefore do not contain 3′ ends typical of eukaryotic mRNAs. Ribosomes do not dissociate from such artificial mRNAs but remain attached to their 3′ ends as a ribosome-mRNA-nascent polypeptide complex [20]. The integrity of this complex is apparently required for posttranslational translocation. It seems possible that this complex imposes restrictions on the tertiary structure of the nascent polypeptide and therefore serves to maintain it in a conformation acceptable for translocation.

The requirement for a functional signal can be interpreted in two ways: either a stretch of hydrophobic amino acids is required simply to trigger interaction between the polypeptide and the membrane of the microsome, or it is required to interact with signal recognition particle (SRP). In the latter case, SRP may serve to increase the efficiency with which the ribosome complex is attached postsynthetically to the microsomal membrane or it may merely place further constraints on the conformation of the nascent polypeptide. We are therefore currently analyzing the dependence of posttranslational translocation of truncated forms of HA on the presence of SRP.

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