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Posttranslational Translocation of Influenza Virus Hemagglutinin across Microsomal Membranes

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The biosynthesis of influenza virus hemagglutinin (HA) and its translocation across microsomal membranes were studied in a mammalian cell-free system. All forms of HA could be cotranslationally translocated with high efficiency. However, only truncated forms of HA were translocated after protein synthesis had been terminated. The efficiency of this posttranslational translocation was dependent on the extent of the truncation. Posttranslational translocation was ribosome dependent and occurred only in the presence of a functional N-terminal signal sequence. The molecular mechanism of protein targeting and translocation across the membrane of the endoplasmic reticulum is discussed.

For many years, it was believed that translocation of secretory and transmembrane proteins into the rough endoplasmic reticulum of eucaryotic cells was strictly coupled to the biosynthesis of the proteins, and chain elongation was seen as providing the driving force for the movement of the nascent polypeptides across the endoplasmic reticulum membrane (2, 3, 15). However, several proteins have recently been reported to be able to cross endoplasmic reticulum membranes after synthesis has been completed. These include three small proteins, i.e., M13 procoat (17), honeybee prepromelittin (19), and frog prepropeptide Gla (14), and precursors to a number of larger proteins, including human glucose transporter (10), human placental lactogen (4), the immunoglobulin G light chain and prolactin (1), and a fusion protein consisting of the β-lactamase or opsin signal region fused to an α-globin (12). Posttranslational translocation is not limited to mammalian cell-free systems, since yeast prepro-α-factor has been shown to enter yeast microsomes after being synthesized in a cell-free system derived from yeasts (7, 13, 16). In every case, posttranslational translocation requires ATP and, with the exception of translocation of prepro-α-factor and the three small proteins, the presence of ribosomes.

We have analyzed the ability of a well-characterized transmembrane protein, influenza virus hemagglutinin (HA), to be translocated co- and posttranslationally across the membrane of the endoplasmic reticulum. Cloned cDNA that encodes the HA of the A/Japan/305/57 strain of influenza virus (5) was inserted into the vector pT7/T3-19 (Bethesda Research Laboratories) so that its coding strand was oriented immediately downstream from the bacteriophage T7 promoter. After linearization with appropriate restriction enzymes, the resulting construct (pT7HA-Jap; Fig. 1) was transcribed in vitro by bacteriophage T7 DNA-dependent RNA polymerase (Bethesda Research Laboratories) for 1 h at 37°C. Trace amounts of [^{32}P]UTP were included in the transcription mixture. Radiolabeled RNA was analyzed by electrophoresis, using formaldehyde-agarose gels, and visualized by autoradiography (9). In every case, over 98% of the RNA was of the correct length and was used in the translation reaction without purification. RNA (0.5 μg) was incubated in a rabbit reticulocyte cell-free system and incubated at 30°C for 40 min according to the instructions of the manufacturer (Promega Biotec) with 2 equivalents of canine pancreatic rough membranes (a gift from Peter Walter, University of California, San Francisco), which were added either before protein synthesis was initiated or after it had been artificially terminated with cycloheximide (0.1 mg/ml, 20 min on ice). Control experiments in which cycloheximide was added before the RNA demonstrated that this concentration was sufficient to totally inhibit protein synthesis (data not shown). In some cases, the translation mixture was treated with proteinase K (0.05 mg/ml) for 25 min on ice in the presence or absence of Triton X-100 (0.5%) before the addition of phenylmethylsulfonyl fluoride (0.5 mg/ml for 20 min on ice). In other cases, puromycin was added to a concentration of 1 mM at the end of the translation reaction. Radiolabeled HA was immunoprecipitated with a polyclonal antiserum that recognizes both native and denatured forms of the protein and then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. The relative amount of radiolabeled protein in each lane was quantitated by densitometric scanning of the X-ray film.

Figure 2 shows the HA polypeptides encoded by RNAs transcribed from various pT7HA-Jap templates and the ability of these polypeptides to be translocated either co- or posttranslationally. The results of typical experiments are shown in Fig. 3 through 5. Full-length HA carrying a wild-type signal (HA_{562}; Fig. 3, lanes 1 to 8) was translocated when rough microsomes were present during biosynthesis of the molecule. The translocated molecules became glycosylated (as judged by the decrease in electrophoretic mobility; lane 2) and resistant to exogenously added protease (lane 3). As expected, disruption of microsomes with Triton X-100 rendered the translocated HA sensitive to protease (lane 4). Because no translocated HA was detected when microsomes were added to the reaction after synthesis of HA had been terminated (lane 5), we conclude that translocation of full-length HA is obligatorily cotranslational in this cell-free system. As expected, cotranslational translocation was dependent on the presence of a functional N-terminal signal sequence, since full-length HA ($HA_{562}S^-$) carrying two mutations in the signal region (Fig. 1B) was not detectably translocated (Fig. 3, lanes 9 to 11). Addition of the reducing

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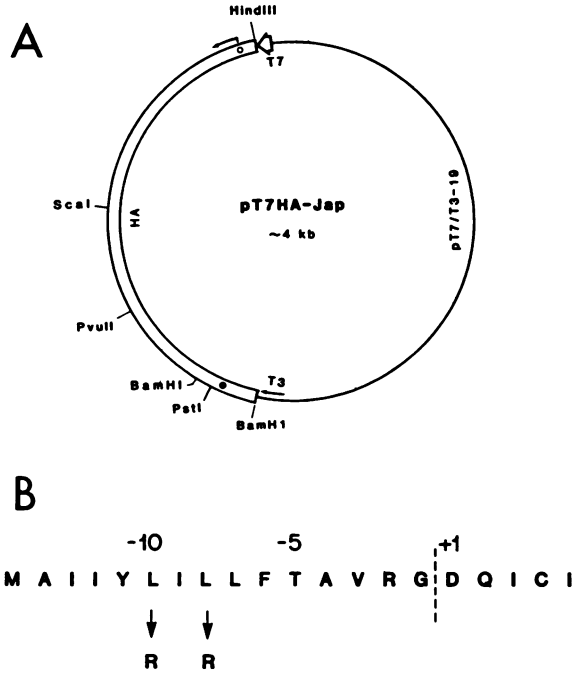

FIG. 1. Restriction map of pT7HA-Jap and the polypeptide sequence of the N-terminal HA signal peptide. Cloned DNA of the Japan strain of HA (5) was inserted either directly into the expression vector pT7/T3-19 (Bethesda Research Laboratories) or into bacteriophage M13 for mutagenesis before it was inserted into the vector. Standard recombinant DNA techniques were used (9). (A) Restriction map of pT7HA-Jap. The restriction sites shown were used to linearize the plasmid for transcription. kb, Kilobases. Symbols: →, direction of transcription; ○, translation initiation point; ●, translation termination point; ⇨, promoter utilized to generate mRNA in vitro. (B) Polypeptide sequence of the N-terminal hydrophobic signal of wild-type HA. The dotted line indicates the cleavage site for signal peptidase. By using oligonucleotide-directed mutagenesis (20), we constructed a mutant form of HA in which leucine residues (L) at positions −8 and −10 of the signal were replaced with arginine residues (R).

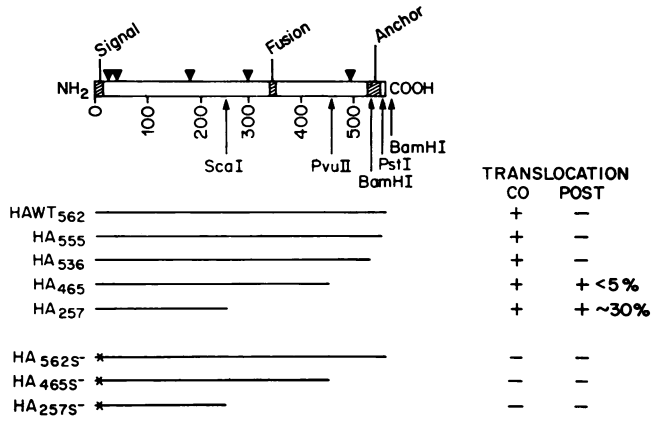

FIG. 2. Translocation of HA synthesized in vitro across microsomal membranes. The locations of five potential glycosylation sites (▼) and three hydrophobic domains (▨) are shown on the diagram of full-length, wild-type HA (top). The scale shows the length of the molecule measured in amino acids. The locations of the restriction sites used to generate linearized forms of pT7HA-Jap are marked (↑): *Bam*HI was used for $HAWT_{562}$ (wild type); *Pst*I, for HA_{555}; *Bam*HI, for HA_{536} (this mutant was derived from a construct in which a *Bam*HI site had been inserted at sequences coding for amino acid 536); *Pvu*II, for HA_{465}; and *Sca*I, for HA_{257}. Plasmids linearized at these sites were transcribed in vitro by T7 RNA polymerase, and the resulting RNAs were tested for the ability to direct co- or posttranslational translocation of HAs across microsomal membranes. A summary of the results obtained is shown on the right. The efficiency of posttranslational translocation was calculated by densitometric scanning of X-ray films.

agent dithiothreitol to the cell-free system did not result in detectable posttranslational translocation of HA (lanes 7 and 8).

By contrast, a truncated form of HA comprising the N-terminal 257 amino acids of the molecule (HA_{257}; 46% of the full length) was translocated both co- and posttranslationally (Fig. 4, lanes 1 to 12). Cotranslational translocation (lane 2) was always much more efficient than posttranslational translocation (lane 5). In the experiment shown in Fig. 4, approximately 70% of HA_{257} was translocated when microsomes were present during the entire period of incubation; however, only 30% of HA_{257} was translocated when microsomes were added to the system after synthesis of HA had been terminated with cycloheximide (lane 5). Nevertheless, the fraction of HA_{257} that was translocated into microsomes posttranslationally was both glycosylated and resistant to protease (lanes 8 to 11). Posttranslational translocation of truncated HA was abrogated both by puromycin (lane 6) and by the presence of a mutated signal sequence (compare lanes 5 and 15). We therefore conclude that this process depends on the integrity of the ribosome-mRNA complex and that the hydrophobic signal responsible for cotranslational translocation is also required for posttranslational translocation.

Comparison of lanes 2 and 5 (Fig. 4) shows differences in the pattern of glycosylation of molecules that have been translocated co- and posttranslationally. That the slower-migrating bands (lanes 2 and 5) correspond to different glycosylated forms of HA_{257} was shown by treating the immunoprecipitated samples with endoglycosidase H (11), which caused all forms of HA_{257} to migrate at the same rate

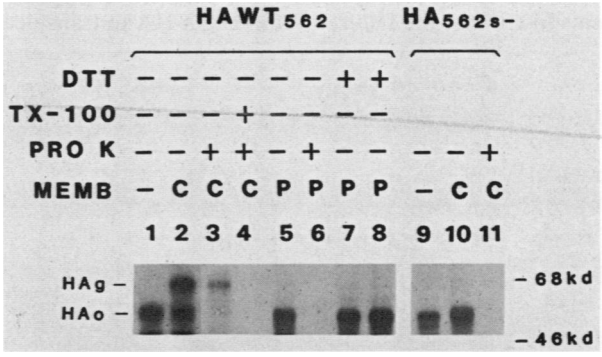

FIG. 3. In vitro translation of HA_{562} cDNA. Lanes: 1 to 8, results of translating RNA transcribed from full-length HA (HA_{562}) carrying a wild-type signal; 9 to 11, results obtained from RNA encoding full-length HA carrying a mutant form of the hydrophobic signal peptide ($HA_{562}S^-$); 7 and 8, HA synthesized in the presence of 5 or 50 mM dithiothreitol (DTT); 4, effect of adding Triton X-100 (TX-100) to the translation mixture, to a final concentration of 0.5%, before addition of proteinase K (PRO K), to a concentration of 50 μg/ml. Canine pancreas rough microsomal membranes (MEMB) were added to the reaction mixtures before protein synthesis was initiated (C) or after synthesis had been terminated with cycloheximide (P). HAo, Nonglycosylated HA; HAg, glycosylated HA; kd, kilodaltons.

as protein synthesized in the absence of microsomal membranes (results not shown). HA_{257} contains three potential sites for the addition of N-linked oligosaccharides (Fig. 2). Most of the cotranslationally translocated molecules of HA_{257} were fully glycosylated. However, the posttranslationally translocated molecules were only partially glycosylated, carrying on average two of the three possible carbohydrate side chains (HAg2 in Fig. 4). Because the ribosome remains associated with the nascent polypeptide in the absence of puromycin, it is possible that the distal glycosylation site (which is less than 90 residues from the carboxy terminus) is inaccessible to glycosyl transferase (6). Alternatively, translocated HA_{257} may assume a conformation which prevents glycosylation at one of the three potential sites (6).

The translocation of a larger form of HA (HA_{465}; 83% of the full-length molecule) was also studied. This truncated molecule was translocated cotranslationally as efficiently as HA_{257} (Fig. 5, lane 2). However, less than 5% of the HA_{465} molecules were translocated posttranslationally (lanes 3 and 5), and longer exposure of the X-ray film was required to detect the band of glycosylated HA (lane 5). This minor population of translocated molecules was resistant to protease (compare lane 5 with lanes 6 and 7). Posttranslational translocation of HA_{465} was dependent on the integrity of the hydrophobic N-terminal signal sequence, since $HA_{465}S^-$ was unable to enter microsomes either co- or posttranslationally (lanes 9 and 10). Because addition of puromycin to the translation reaction completely prevented the translocation of HA (compare lanes 3 and 4), we conclude that integrity of the ribosome-mRNA-tRNA-polypeptide complex is required for posttranslational translocation. Truncated molecules of HA (e.g., HA_{536} and HA_{555}) that are greater in length than 83% of the wild-type molecule were unable to translocate posttranslationally across microsomal membranes (data not shown).

These results raise several questions. For example, why should posttranslational translocation be restricted to molecules of HA that are less than full length? Why should the process require both a functional signal sequence and integrity of the ribosome-mRNA-nascent protein complex? It seems likely that the failure of full-length HA to translocate

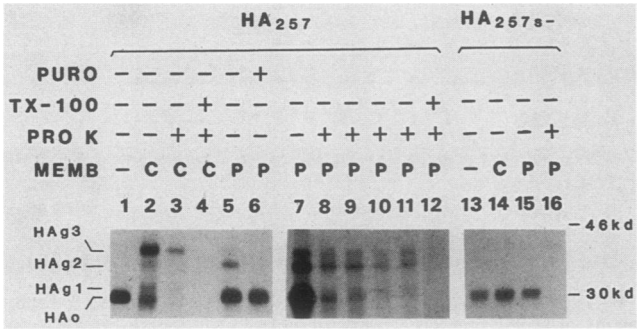

FIG. 4. In vitro translation of truncated HA_{257} cDNA (46% of the full-length molecule). RNA encoding HA_{257} (lanes 1 to 12) or $HA_{257}S^-$ (lanes 13 to 16) was translated in vitro. The resulting HA molecules were treated with proteinase K (PRO K) for 0, 5, 10, 30, or 60 min (lanes 7 to 11, respectively). Lane 6 shows the effect of adding puromycin (PURO) after translation of HA_{257} RNA and before addition of canine pancreas rough microsomal membranes (MEMB). HAo, HAg1, HAg2, and HAg3 mark the positions of HA_{257} molecules carrying zero, one, two, or three oligosaccharide groups, respectively. The other abbreviations are described in the legend to Fig. 3.

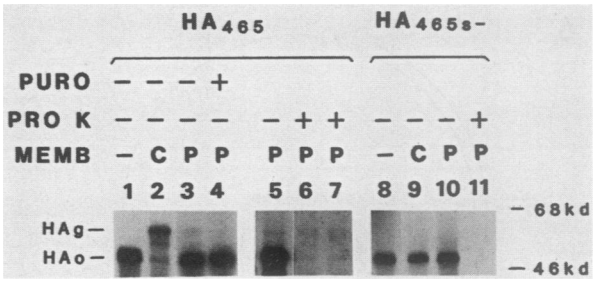

FIG. 5. In vitro translation of truncated HA_{465} cDNA (83% of the full-length molecule). RNA encoding HA_{465} (lanes 1 to 7) or $HA_{465}S^-$ (lanes 8 to 11) was translated in vitro. The samples shown in lanes 5 through 7 were treated with proteinase K for 0, 25, or 60 min, respectively. The symbols are described in the legends to Fig. 3 and 4.

is a consequence of the protein's folding into a conformation that is incompatible with its transfer across the microsomal membrane. This hypothesis has been proposed previously by Maher and Singer (8), who suggested that the formation of disulfide bonds in preprolactin synthesized in cell-free systems was sufficient to prevent its posttranslational translocation. However, this explanation does not fit our observations, since the inclusion of reducing agents in the in vitro system did not facilitate posttranslational translocation of the full-length HA (Fig. 3, lanes 7 and 8). Even in the absence of disulfide bond formation, it therefore seems that wild-type HA can fold into a configuration(s) that is unacceptable for translocation. However, HA_{257}, which lacks 305 amino acids from the carboxy terminus, displays considerable ability to be translocated posttranslationally. Presumably, the efficiency of this process reflects the proportion of the truncated molecules which have avoided forming structures that cannot be translocated posttranslationally. HA_{257} differs from the wild-type protein and the longer mutants in lacking the sequences that form the long alpha-helical domains in correctly folded, trimeric HA (18). If these long helices form in monomeric HA synthesized in vitro, they may prevent the posttranslational translocation of the molecule.

The mRNAs that encode truncated forms of HA (Fig. 2) were generated by in vitro transcription of linearized plasmids and therefore do not contain 3′ termini typical of eucaryotic mRNAs. Ribosomes do not dissociate from such artificial mRNAs, but remain attached to their 3′ ends (1). The integrity of this complex is apparently required for posttranslational translocation. Therefore, it seems possible that attachment to this complex imposes restrictions on the tertiary structure of the nascent polypeptide and serves to maintain it in a conformation acceptable for posttranslational translocation.

The requirement for a functional signal sequence can be interpreted in two ways: either a stretch of hydrophobic amino acids is required simply to trigger the interaction between the polypeptide and the membrane of the microsome, or it is required to interact with a signal recognition particle. In the latter case, the signal recognition particle 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 signal recognition particles.

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