Role of signal recognition particle in the membrane assembly of Sindbis viral glycoproteins

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We have investigated the role of signal recognition particle (SRP) in the biosynthesis of Sindbis glycoproteins by translating the viral 26S mRNA in a wheat-germ cell-free system. SRP was shown to have no effect on the synthesis or proteolytic processing of the cytoplasmic C protein. In contrast, the membrane integration and the proteolytic processing of the viral glycoproteins PE_2 and E_1 were demonstrated to be SRP-dependent. In the absence of microsomal membranes, SRP caused an arrest of the synthesis of the viral glycoproteins. This arrest could be released by the addition of salt-extracted microsomal membranes. Synchronization experiments indicated that the uncleaved signal sequence of PE_2 was recognized by SRP after at most 130 amino acids of PE_2 had been polymerized. No apparent interaction of SRP with a putative signal sequence of E_1 and/or a 6-kDa peptide could be detected.

For several representative examples, it has recently been demonstrated that the mechanism of integration into the rough endoplasmic reticulum membrane of transmembrane proteins [1] and the mechanism of complete translocation across the same membrane of secretory [2-4] and lysosomal [5] proteins have specific steps in common. Signal recognition particle (SRP), an 11-S ribonucleoprotein [2,6], was found to interact directly with ribosomes which are in the process of translating mRNAs coding for these proteins [7] and to target them to the endoplasmic reticulum membrane [8]. On free ribosomes, SRP caused a site-specific elongation arrest of proteins expressing a signal sequence on their nascent chains [9], that was only released after the arrested ribosome interacted with a specific integral membrane protein of the endoplasmic reticulum (the SRP receptor [9-11] or docking protein [3,12]). Having thus accomplished correct targeting, the nascent chain is subsequently vectorially translocated across the membrane.

We decided to study the role of SRP in the biosynthesis of Sindbis viral proteins. The biosynthesis of Sindbis (as well as the closely related Semliki forest virus) proteins poses interesting questions because a single viral mRNA (26S RNA) encodes one cytoplasmic (C protein, 30 kDa) [13] and three distinct membrane proteins (PE₂ of 60 kDa, a 6-kDa peptide and E₁ of 53 kDa) [13] in this order (for a recent review see [14]). These proteins are thought to be generated via a series of cotranslational endoproteolytic processing steps.

The biosynthesis, core glycosylation and membrane assembly of Sindbis and Semliki forest viral glycoproteins (PE₂ and E₁) have been faithfully reproduced *in vitro* using cell-free translation systems supplemented with microsomal membranes [15,16]. These studies revealed that translation of 26S RNA initiated and proceeded on free ribosomes. As soon as C protein was completed, it was found to be cleaved from the nascent chain. The translating ribosomes then attached to the microsomal membranes, resulting in cotranslational membrane integration and proteolytic processing to yield PE_2 , the 6-kDa peptide and E_1 . The signal sequence of PE_2 was shown not to be cleaved off during membrane integration [17]. Based on data from synchronized translation systems [16] and from sequence analysis [13], it has been suggested that the uncleaved signal sequence of PE_2 is located near its amino terminus. Sequence data also suggested that the 6-kDa peptide might function, at least in part, as a signal sequence for the membrane integration of E_1 [18].

In the absence of microsomal membranes, the translation of 26S RNA resulted in the synthesis of C protein and a 110kDa protein, named B_1 , which was found to be a polyprotein comprising the primary sequences of PE₂ plus the 6-kDa peptide plus E₁. Thus, the proteolytic conversion of B₁ to PE₂, the 6-kDa peptide and E₁ was dependent on the presence of microsomal membranes in the translation system [15]. Here we demonstrate that SRP recognizes the nascent Sindbis B₁ protein via the uncleaved signal sequence in PE₂ in a manner analogous to that found for secretory and other integral membrane proteins possessing cleaved signal sequences. SRP has no apparent effect on C protein synthesis and apparently does not recognize the 6-kDa peptide in nascent B₁ as a signal sequence.

MATERIALS AND METHODS

All the procedures used in this paper have been described in detail elsewhere. They include: isolation of an RNA fraction enriched in Sindbis 26S RNA from infected chick embryo fibroblasts and the conditions for translation in a wheat-germ cell-free protein-synthesis system [19]; the preparation of saltextracted microsomal membranes from dog pancreas rough

Abbreviations. SRP, signal recognition particle; PE_2 and E_1 , viral glycoproteins; B_1 , a polyprotein.



Fig. 1. Effect of SRP on the synthesis of Sindbis viral proteins. 26S RNA was translated in the wheat-germ cell-free protein-synthesis system in the absence (lanes 1, 2 and 7) or in the presence of 52 units/ml (lane 3), 260 units/ml (lanes 4 and 6), 520 units/ml (lane 5) of SRP. Salt-extracted dog pancreas microsomal membranes were present at a concentration of 80 equivalents/ml [2] in the incubation mix (lanes 6 and 7). In lane 1 no 26S RNA was added. All incubations were performed at 27°C for 150 min in the presence of 59 MBq/ml of [³H]eucine. 12-µl aliquots of each incubation mix were analyzed on a 10-15% gradient polyacrylamide slab gel. After impregnation with PPO [20], the dried gel was fluorographed for 48 h. The arrow indicates a minor 14-kDa translation product which is synthesized concomitant to the 8-kDa polypeptide

microsomes and the purification of SRP [2]; polyacrylamide slab gel electrophoresis and subsequent fluorography [17,20]. To quantify the radioactivity incorporated in specific polypeptides. bands were located by fluorography, excised and rehydrated. PPO was removed by repeated washing in dimethylsulfoxide and the bands were cut into small pieces and incubated for 48 h at 55 °C in 1 ml 30% H₂O₂ containing 20 µl of 1 M HCl. After cooling, the radioactivity was determined by liquid scintillation counting in 9 ml of Dimilume.

RESULTS AND DISCUSSION

To assess the role of SRP in the biosynthesis of Sindbis glycoproteins we translated viral 26S RNA in a wheat-germ cell-free system. As previously demonstrated [17], we obtained two major translation products (Fig. 1, lane 2): C protein (30 kDa) and B₁ protein (110 kDa). Upon addition of increasing amounts of purified SRP to the translation system (Fig. 1, lanes 3-5) we observed a specific inhibition of B₁ protein synthesis, weheras the synthesis of cytoplasmic C protein was not affected. A quantitative interpretation of these data (Fig. 1, lanes 2-5) is given in Fig. 2. Interestingly, concom-



Fig. 2. *SRP inhibits synthesis of B*₁ *protein but not of C protein.* The amount of C and B₁ proteins synthesized in the experiment shown in Fig. 1, lanes 2-5, was determined as detailed in Methods. The amount of radioactivity found in gel slices of equal size in translation performed in the absence of 26S RNA was subtracted as background. The ³H cpm were normalized to be 100 for C and B₁ synthesized in the absence of SRP

itant with the inhibition of B_1 protein synthesis, we observed the appearance of a new small-molecular-mass translation product, a major band at 8 kDa. By analogy with the known mechanism of the SRP-induced inhibition of secretory and membrane protein synthesis, it appears likely that the lowmolecular-mass translation product represents an elongationarrested form of B_1 . However, a conclusive assignment has to await sequence determination.

Addition of salt-extracted (SRP-depleted) microsomal membranes to the SRP-arrested translation system (conditions as in Fig. 1, lane 4) resulted in the disappearance of the 8-kDa polypeptide as well as residual B_1 and a concomitant appearance of PE_2 (Fig. 1, lane 6). PE_2 was previously demonstrated to arise as a proteolytic cleavage product upon proper membrane integration of nascent B₁ in systems reconstituted in vitro. The synthesis of the second cleavage product (E_1) is not efficient in the wheat-germ translation system in vitro (presumably due to a poor translation efficiency of the distal portions of the mRNA). However, upon longer exposure of the gels, bands equivalent in migration to the previously reported translation product in the more efficient reticulocyte lysate system were detected (data not shown). We therefore did not detect E_1 protein synthesis under the conditions where PE_2 protein was generated (Fig. 1, lane 6).

In contrast to a translation system supplemented with translocation-competent native microsomal membranes [15] or salt-extracted microsomal membranes plus SRP (Fig. 1, lane 6), the addition of salt-extracted microsomal membranes alone (in the absence of SRP) had no qualitative effect on the pattern of translation products (Fig. 1, compare lanes 2 and 7). Thus both membrane integration of B_1 and its subsequent proteolytic conversion to PE_2 (and E_1) were dependent on SRP.

To address the question of whether SRP would also recognize the putative second signal sequence (suggested to be responsible for E_1 integration), we added SRP to a synchronized translation system. Translation of 26S RNA was allowed to initiate for 3 min, after which time 7-methylguanosine 5'-monophosphate was added to prevent further initiation. Incubation was continued for 150 min. Since this treatment does not affect elongation, it allows one to observe the fate of the nascent polypeptide chain as a function of time.



Fig. 3. Function of SRP and of translocation-competent microsomal membranes in a synchronized translation system. 26S RNA was translated in the wheat-germ system. After 3 min, 7-methylguanosine 5'-monophosphate was added to 2 mM to inhibit initiation. At different time points (expressed in min and indicated on the top of each lane), aliquots of the incubation mixture were supplemented with SRP to a final concentration of 260 units/ml (A) or with the same amount of SRP plus 100 equivalents/ml of salt-extracted microsomal membranes (B). Incubation was performed at 27 °C for 150 min in the presence of 59 MBq/ml of [³H]leucine. 10-µl aliquots at each time point were analyzed on a 10-15% gradient polyacrylamide slab gel. After impregnation with PPO the dried gel was fluorographed for 7 days



Fig. 4. Quantification of B_1 and PE_2 proteins synthesized in the experiment shown in Fig. 3. (•) B_1 protein synthesized in the presence of SRP (Fig. 3A); (•) B_1 protein synthesized in the presence of translocation-competent microsomal membranes (Fig. 3B); (•) PE_2 synthesized in the presence of translocation-competent microsomal membranes (Fig. 3B). The radioactivity in the B_1 region at the 5-min time point and in the PE₂ region at the 50-min point was subtracted as background. The ³H cpm were normalized to be 100 for B_1 synthesized at the 50-min time point in both conditions, and for PE₂ glycoprotein synthesized at the 5-min time point

Under our conditions (elongation rate 11 amino acids/min and assuming it to be constant during the entire period of chain elongation), it took 30 min to complete all C protein molecules $(t_{\frac{1}{2}}=26 \text{ min})$ (data not shown). We then added purified SRP (Fig. 3A) or SRP plus salt-extracted microsomes (Fig. 3B) at various times of elongation (indicated at the top of Fig. 3) to the synchronized translation systems. It is apparent from the data in Fig. 3, and their quantitative interpretation in Fig. 4, that SRP will cause elongation-arrest of B₁ or, as in the presence of salt-extracted microsomal membranes, conversion of B₁ to PE₂ (and E₁), only when SRP is present within the first 35 min of translation ($t_{\frac{1}{2}}$ of B₁ inhibition or PE₂ production was 37 min, see Fig. 4). At this time C protein synthesis was completed (see above) and B₁ synthesis had just started (at most, 130 amino acids of B₁ were polymerized). A later addition of SRP (Fig. 3 and 4) had no effect and the translation products obtained were essentially identical to those obtained in the absence of SRP (Fig. 1, lane 2).

The data in Fig. 1 and 2 clearly show that SRP recognized the uncleaved signal sequence on PE_2 . The size of the putative SRP-arrested fragment (8 kDa) (Fig. 1), as well as kinetic data of the synchronization experiments (Fig. 3 and 4), are consistent with the previously suggested localization of the signal sequence to the amino-terminal portion of PE_2 [13, 16].

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