The Signal Sequence of the p62 Protein of Semliki Forest Virus Is Involved in Initiation but Not in Completing Chain Translocation

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Abstract. So far it has been demonstrated that the signal sequence of proteins which are made at the ER functions both at the level of protein targeting to the ER and in initiation of chain translocation across the ER membrane. However, its possible role in completing the process of chain transfer (see Singer, S. J., P. A. Maher, and M. P. Yaffe. Proc. Natl. Acad. Sci. USA. 1987. 84:1015–1019) has remained elusive. In this work we show that the p62 protein of Semliki Forest virus contains an uncleaved signal sequence at its NH2-terminus and that this becomes glycosylated early during synthesis and translocation of the p62 polypeptide. As the glycosylation of the signal sequence most likely occurs after its release from the ER membrane our results suggest that this region has no role in completing the transfer process.

Biosynthesis of proteins at the ER can be subdivided into several steps. These are (a) targeting of translation complexes to the ER membrane; (b) synthesis and transfer (translocation) of the polypeptide chain across the lipid bilayer; and (c) protein maturation in the lumen of ER (chain folding, disulphide bridge formation, glycosylation, and oligomerization). The mechanisms for these processes have been studied extensively during recent years (Kornfeld and Kornfeld, 1985; Wickner and Lodish, 1985; Rapoport, 1986; Lodish, 1988; Rothman, 1989). A most important finding has been that all proteins made at the ER carry a signal sequence (also called signal peptide), a hydrophobic peptide which is usually located at the NH2-terminal region of the polypeptide chain. One function of the signal peptide is to achieve targeting of the polysome to the ER membrane (Rapoport, 1986). When the signal sequence emerges from the ribosome it binds to the signal recognition particle, which mediates binding of the polysome to the docking protein in the ER. After this another function of the signal sequence is expressed, that is to interact with some components of the ER membrane and thereby initiate translocation of the polypeptide chain into the lumen of the ER (Gilmore and Blobel, 1985; Robinson et al., 1987; Wiedmann et al., 1987). Further synthesis of the polypeptide then continues with concomitant chain translocation. An important but as yet unresolved question is whether the signal sequence has any role in the translocation process per se or whether its functions are limited to the targeting and translocation-initiation steps. For instance, Singer and co-workers (1987a) have suggested a translocator protein model in which the signal sequence helps to keep the machinery open for chain transfer.

It is specifically this last question we have addressed in the present work. We describe the characteristics and behavior of the uncleaved signal sequence of the p62 protein of Semliki Forest virus (SFV) upon translocation across the ER membrane in vitro. The p62 protein is one subunit of the heterodimeric spike protein of the SFV membrane (reviewed in Garoff et al., 1982). It is made as a precursor protein together with the other structural proteins of SFV, i.e., the nucleocapsid protein, C, and the other spike subunit, E1. The three proteins are synthesized from a 4.1-kb long mRNA in the order C, p62, and E1, and separated by cleavage of the growing precursor chain. During synthesis of the p62 polypeptide at the ER all but a 31 residue COOH-terminal portion and the membrane anchor is translocated across the membrane. The p62 signal sequence has so far been only roughly localized to the NH2-terminal third of the polypeptide chain. We show here that the signal sequence of p62 consists of a 16 residue peptide at its NH2-terminal region. This region includes one out of four glycosylation sites (Asn11) for N-linked oligosaccharide on the p62 chain. We also demonstrate that the glycosylation of the p62 signal sequence occurs early during chain translocation. As this modification of the signal region most likely correlates with its release into the lumen of ER it follows that the signal sequence of p62 is probably only needed for an initial step in chain translocation and not to complete the process.
maintain the competence of the chain translocation machinery throughout p62 chain transfer.

**Materials and Methods**

**Materials**

T4 Poly-nucleotide kinase, SP6 Polymerase, and most restriction endonuclease class were obtained from Boehringer Mannheim Biochemicals (Mannheim, FRG), except for Xmn I and Sac I (New England Biolabs, Beverly, MA). T4 DNA Polymerase was purchased from Bethesda Research Laboratories (Rockville, MD), RNase inhibitor from Promega Biotech (Madison, WI), protease K from E. Merck (Darmstadt, FRG), and Xho I linker (dCCCTCAGGG) from Collaborative Research (Lexington, MA). T4 DNA ligase was a gift from W. Fiers (Laboratory of Molecular Biology, State University of Ghent, Belgium) and purified from an E. coli strain that thermoinduces overproduces this enzyme from a bacterial expression vector (Renaut et al., 1983). Ribonucleotides (from equine muscle) and spermidine used for RNA synthesis, together with PMSF, diethyl pyrocarboxylate (DEPC), and BSA (fraction V) were from Sigma Chemical Co. (St. Louis, MO). The cap analogue mG3p(jpG)5pG and protein A-Sepharose CL-4B were from Pharmacia (Freiburg, FRG). RNase inhibitor was from Promega Biotech. Methylated, 14C-labeled proteins were used as molecular mass markers and Enhance (for fluorography) were from DuPont de Nemours (NEN Research Products, Dreieich, FRG). Reticulocyte lysate was prepared from rabbits according to established procedures (Jackson and Hunt, 1983). Some lysate of the same kind (N.150) was also purchased from American International (Amersham, UK) together with stabilized L-[35S]methionine (SI.151). Dog pancreas rough microsomes (50 A260/ml) were partly provided by B. Dobberstein (European Molecular Biology Laboratory) and partly prepared by ourselves according to Kaderbai and Austen (1984). The rabbit antimonus dfr antibody was a generous gift from E. Hurt (European Molecular Biology Laboratory). DNA fragments were purified in LMP agarose (Bethesda Research Laboratories). The acceptor peptide N-benzoyl-Asn-Leu-Thr-N-methylaspartic acid and the alphalohion (siThr) containing nonacceptor peptide N-benzoyl-Asn-Leu-Asn-Thr-N-methylaspartic acid were synthesized according to Erickson and Merrifield (1976; see also Tillmann et al., 1987). Aurintricarboxylic acid (ATA) was from Sigma Chemical Co.

**General DNA Methods**

Small scale plasmid DNA preparations were done using the alkali-SDS method essentially as described by Birnboim and Doly (1979). Large quantities of plasmids to be used for in vitro transcription were prepared by lysosome-Triton lysis of the bacteria, followed by CsCl-EtBr banding (Kahn et al., 1979). E. coli was removed by several extractions with isopropanol and, after fivefold dilution, the DNA was precipitated twice with ethanol and further purified over a Biorad A-50m column. Restriction endonuclease and DNA-modifying enzymes were used according to the suppliers instructions. Removal of the 3' sticky end from the Sac I site in pGEM2-alphaG (Zerial et al., 1986) with T4 DNA Polymerase was done at 15°C (2 h), dNTPs were added (final concentration 100 #M each), and the DNA was subsequently filled in at 15°C for 1 h. All ligations were done at 24°C for 4 h except for linker ligations (4°C, 16 h). All other molecular biological manipulations were done using slightly modified standard protocols (Maniatis et al., 1982).

**In Vitro Transcription and Translation**

In vitro transcription (0.3 #g supercoiled template DNA per 10 #l vol) in the presence of SP6 RNA Polymerase (6-8 U) and the cap structure was carried out as previously described (Zerial et al., 1986). In vitro transcription reactions using a rabbit reticulocyte lysate were performed at 30°C essentially as described (Melancon and Garoff, 1986). 1.5 #g of the in vitro synthesized RNA was translated in a total volume of 15 #l. Potassium, magnesium, and spermidine concentrations were 100, 1,2, and 0.375 mM, respectively. When indicated, 1 #l of ER membranes was included. In some translations the membranes were pretreated with 200 #M peptide for 5 min on ice. The final peptide concentration in the translation mixture here was, after addition of the pretreated membranes, adjusted to 100 #M. To obtain optimal synchronisation of translation, ATA was added after a preincubation of 1.5-5.0 min (Borgese et al., 1974). A final ATA concentration of 0.075 mM was found to be sufficient for initiating chain synthesis (see control in Fig. 6, lane J). Higher concentrations of ATA inhibited first translation and then also chain elongation. For protease protection experiments, protease K was added to a final concentration of 0.1 mg/ml and the samples were incubated at 0°C for 30 min in the presence or absence of 1% Triton X-100. Proteolysis was stopped by the addition of PMSF (final concentration 2 mg/ml) and samples were kept at 0°C for 5 min before further processing for electrophoresis (Cutler and Garoff, 1986). Bands containing labeled protein were visualized by fluorography. Quantitation of proteins was done by cutting the bands out of the dried gel, solubilizing them with Protosol (from DuPont de Nemours, NEN) according to the instructions of the manufacturer, and finally counting the 35S radioactivity in a liquid scintillator (Wallac IKB, Turku, Finland). The localization of the bands on the dried gel was done with the aid of the fluorograph in transillumination.

**Alkaline Treatment of Cell-free Translation Mixtures**

15-#l translation mixtures were adjusted to pH 11-11.5 by adding an appropriate volume (pretitrated) of 0.1 N NaOH. After a 10-min incubation on ice the samples were separated into a pellet fraction and a supernatant fraction by centrifugation through a 100-#l alkaline sucrose cushion (Gilmore and Blobel, 1985) for 10 min at 30 psi in an airfuge (Beckman Instruments, Inc., Palo Alto, CA) using a A-100/30 rotor and cellulose propionate tubes precoated with BSA (1% solution). The entire supernatant was removed, neutralized with 1 N HCl, diluted 2.5 times with water, and then precipitated by adding 3.5 vol of acetone. These precipitated proteins and pelleted membranes (obtained from the airfuge tubes) were taken up in 4% SDS by incubating at 56°C for 15 min and then processed for immunoprecipitation reactions as described below.

**Immunoprecipitation of Fusion Protein from Reticulocyte Lysate**

Total translation mixtures were adjusted to 4% SDS, then boiled for 4 min and diluted 1:2 with water. 4 vol of immunoprecipitation buffer (2.5% Triton X-100, 190 mM NaCl, 60 mM Tris-HCl, pH 7.4, 6 mM EDTA, and 20 #g PMSF/ml) and 2 #l of antibody were added for 16 h at 4°C. The mixture was briefly centrifuged (2-3 min in an Eppendorf minifuge) and to the supernatant one fifth volume of a 1:1 slurry of protein A beads were added and incubated at 24°C for 2 h under constant agitation. The beads were collected and washed four times with 1 ml RIPA buffer (Gielkens et al., 1976) by centrifugation, followed by a single wash with a buffer containing 150 mM NaCl, 10 mM Tris-HCl pH 7.4, and 20 #g PMSF/ml. The beads were then taken up in excess gel loading buffer (Cutler and Garoff, 1986), heated at 95°C for 5 min, and cleared by centrifugation before loading the immunoprecipitate on the gel.

**Plasmid Constructions**

Constructions of pGEM2alphaGX and pGEM2dfrX. For the construction of the final fusion protein-coding plasmids used in this study we first had to make plasmids pGEM2alphaGX, which are derived from pGEM2alphag and pGEM2dfrX, which are derived from pGEM2dfr (Zerial et al., 1986). Plasmid pGEM2alphaGX contains a 548 bp-long Nco I-Pst I fragment encompassing the entire chimpanzee alpha-globin coding region between the Hinc II and Pst I sites of the polylinker of the plasmid pGEMI (Promega Biotech). The Nco I site contains the translation initiation codon from alpha-globin (Zerial et al., 1986). An Xho I site, allowing subsequent in-frame ligations of SVF sequences, had to be introduced in pGEM2alphag. Therefore, this plasmid was cut (upstream of the Nco I site) with Sac I, the 3' sticky ends removed with T4 DNA Polymerase, an Xho I octamer linker introduced and, after cutting with Xho I, the plasmid was religated at low DNA concentration (1 #g/ml). Plasmid pGEM2alphaGX then contains the 2,057 bp-long Xho I-Pvu I fragment needed for the construction of the fusion protein-coding plasmid pGEM2alphaG.

An intermediate construct, analogous to pGEM2alphaGX, and also containing the unique Xho I site, was needed for the constructions of dfr-containing plasmids. For this purpose we inserted the Xho I linker into partially Xmn I cut pGEM2dfr (Zerial et al., 1986). After cutting the linkers, linear plasmid was purified on agarose gel and religated. Since the second Xmn I site in pGEM2dfr is located in the beta-lactamase coding region of the vector (Sutcliffe, 1979) and insertion of an Xho I site by an octamer linker will result in an ampicillin-sensitive E. coli phenotype after transformation, only the desired pGEM2dfrX construct was obtained. From this plasmid, an Xho I-Pvu I fragment of at least 2.012 bp (the precise length
Construction of the Fusion Protein-coding Plasmids pC62alphaG and pC62dhfr. Plasmid pGEMI-SFV (also called pG-SFV-15/5; Melancon and Garoff, 1986) contains a reengineered cDNA copy of the SFV 26S mRNA sequence cloned as a Bam HI fragment in the Bam HI site of the polyclinker downstream of the SP6 promoter in the plasmid pGEMI (Promega Biotech). From the SFV plasmid, a 2,381 bp-long Pvu I-Xho I fragment, containing the coding sequences for the capsid protein and the NH2-terminal region of the p62 protein, was isolated. The Xho I-Pvu I fragments from pGEMI-SFV, pGEMalphaGX and pGEMdhfrX were isolated and ligated to the starting vectors by restriction analysis.

The two oligonucleotides were hybridized (51 complementary bases), filled in using Sequenase (United States Biochemical Co., Cleveland, OH) and restricted with Eco RI and Xho I. The resulting DNA fragment was then purified and inserted into pCp62dhfr instead of the C and p62 sequences. The two oligonucleotides were made (DNA-synthesizer; Applied Biosystems, Foster City, CA): (1) 5' ATACACAGAATTCAACCACTGTTCTCCGCCCCGTGGATTACAATCGCATGTGTTCTGATCTTTGCGCAACTCTTCCCCGTGCTTACCGCCCGCTGTTACCTTGC, (2) 5' GTTATCTCGAGCATTCAGTGTGGCCTCTGCGTTGTTTTCATAGCAGCAAGGTACACACGGGGGCTGGAAGCAGGGGAAGGTAGCTATAC.

Localization of the Signal Sequence of p62

To define the p62 signal sequence we have studied the translocation phenotype of two reporter molecules, the rabbit alpha-globin and the mouse dihydrofolate reductase (dhfr), both of which have been extended at their NH2-termini with an NH2-terminal 40 residue peptide from p62. The hybrid molecules were tested in a microsome-supplemented in vitro translation system. The alpha-globin and the dhfr have earlier been shown to be translocation incompetent if not extended with a heterologous signal sequence at their NH2-termini (Zerial et al., 1986).

We first tested the expression of in vitro-made RNA from the construction pCp62dhfr in an in vitro translation system. This would be expected to yield free C protein and p62-reporter hybrid (p62-dhfr) through C-catalyzed autoproteolytic cleavage of the nascent C-p62-reporter precursor (Fig. 1) (Aliperti and Schlesinger, 1978; Hahn et al., 1985; Nunberg et al., 1982). Oligosaccharide residues 1-40 of p62. Oligo 1 (the coding strand) includes, in addition, the region coding for initiator methionine of the C protein plus its 5' flanking sequences (5' AGCACCATG). At the extreme 5' end of this oligo we have added the recognition sequence for Eco RI and its flanking sequences from the 5' end of the structural part of the SFV cDNA (5' ATACACAGATTC). Oligo 2 ends at its 3' end with the Xho I site which follows the signal sequence region on the p62 gene.

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Construction of pGEM SFV d-4. This plasmid was constructed by ligating three fragments together. The first one was the major part of pGEMI, cut just after the promoter region with Hind III and Bam HI. The second fragment (Hind III-Xho I) was isolated from the plasmid p8VS-SFV (Kondor-Koch et al., 1983). This fragment contains the sequences encoding the caspid and the NH2-terminal part of the p62 protein of SFV. The third fragment was obtained by cleaving plasmid pL1 SFV d-4 (see below) with Xho I and Bam HI and isolating the fragment containing the 3' part of the coding sequence for the p62 protein. However, it should be noted that in the d-4 version there is an exchange of 15 codons at the 3' end of the p62 gene for six aberrant ones. The corresponding p62 protein variant is called p62 d-4 (see Fig. 1). It should also be mentioned that pL1 SFV d-4 has been derived from pL1 SFV d-9 (Cutler and Garoff, 1986) by exchanging the Xho I-Cla I region containing the 3' part of the p62 coding region with the similar fragment from pSV2 SFV d-4. This latter plasmid is described in Garoff et al. (1983).

Results

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Figure 1. Composition of polypeptides coded for by plasmid pC62dhfr, pC62-globin, p62dhfr, and pGEM SFV d-4. Numbers refer to amino acid residues as deduced from the known cDNA sequence of chimpanzee alpha-globin (Liebhaber and Begley, 1983), mouse dhfr (Chang et al., 1978; Nunberg et al., 1980; Crouse et al., 1982), and SFV (Garoff et al., 1982). Oligosaccharide residues (CHO) is shown to be linked to Asn residue 13 in the p62 part (Garoff et al., 1982). Additional amino acids resulting from in frame translation of the multicloning region of pGEM2 and the added Xho I linker as well as the initiator Met of p62dhfr are also indicated.
Figure 2. Translation in vitro of p62-dhfr. Plasmid pCp62dhfr was transcribed into RNA and this was used for in vitro translations in the presence (M) or absence of membranes. The samples were analyzed by SDS-PAGE (10%), followed by autoradiography. When indicated, an acceptor peptide (Acc) for N-linked glycosylation or a nonacceptor peptide (Non) was included in the translation. P and TX codes are used when in vitro translations have been treated with protease (P) in the presence (TX) or absence of Triton X-100. Bands corresponding to the C, the p62-dhfr, and glycosylated p62-dhfr proteins are indicated.

Figure 3. Immunological identification of the p62-dhfr hybrid protein and analysis of its association with membranes. RNA transcribed from pC62-dhfr was translated in vitro in the presence of membranes which in some cases had been treated with an acceptor (Acc) or nonacceptor (Non) peptide. The samples were treated, after translation, at pH 11-11.5, and the proteins then separated into a membrane-bound pellet fraction (P) and a supernatant fraction (S) by centrifugation. In all samples the p62-dhfr polypeptides were isolated using an anti-dhfr antibody. The proteins were then analyzed by SDS-PAGE (10%) and subsequent autoradiography. The slower migrating band corresponds to glycosylated and the faster one to nonglycosylated forms of p62-dhfr (compare Fig. 3).

Figure 4. Translation in vitro of p62-dhfr. Plasmid p62dhfr was transcribed into RNA and this was used for translation as described in the legend to Fig. 2.

In the presence of membranes (lane 1) two major protein species were translated from the SP6-directed transcript. One of these had the expected size of C (33 kD) and the other one that of the p62-dhfr hybrid molecule (21 kD). The coding region has apparently been translated faithfully and the precursor protein cleaved efficiently. The identity of the p62-dhfr was directly proven by immunoprecipitation with a dhfr specific antiserum (see Fig. 3). The two weaker bands migrating faster than the capsid in Fig. 2, lane 1 were most likely derived from C coding sequences because they are found in all protein analyses of in vitro transcriptions involving cDNAs with C regions (compare Fig. 6).

When microsomes were added to the C-p62-dhfr in vitro translation system a new band appeared which migrated somewhat slower than the p62-dhfr band seen in the analysis of the mixture lacking membranes (Fig. 2, lane 2). It almost comigrated with one of the two weak C derived bands. The new band apparently corresponds to p62-dhfr hybrids that have been translocated into the lumen of the added microsomes and have become glycosylated. The immunoprecipitation analysis shown in Fig. 3 confirmed the identity of this material. The protease digestions in the absence (Fig. 2, lane 3) and presence of Triton X-100 (lane 4) clearly demonstrated that the slower migrating p62-dhfr molecules were indeed translocated. About half of this material remains protected in the presence of intact microsomes whereas all is digested when the membranes are solubilized with detergent. In contrast, the other translated material did not show such a pronounced membrane-dependent protease resistance. Note that protease treatment of all samples yielded a resistant protein of a small size. This most likely represents a protease-resistant C fragment.

The glycosylation of the translocated p62-hybrid and its effect on the apparent size of the protein was shown in an experiment where a short peptide (Asn-Leu-Thr), which competes for N-linked glycosylation, was included during translation. Apparently only unglycosylated faster migrating p62-dhfr hybrids were formed in these conditions although chain translocation took place conferring protease resistance (Fig. 2, lanes 5-7). Additional analyses (lanes 8-10) illustrate that a control peptide (Asn-Leu-αThr) which cannot serve as an acceptor site for N-linked glycosylation, had no effect on the glycosylation of the p62-reporter hybrids when tested in an analogous way.

Similar studies as with pCp62dhfr were also performed with the pCp62globin coded proteins in vitro. The results (not shown) were analogous to those described above for the pCp62dhfr construct. C protein and p62-globin hybrid were synthesized in the absence of membranes. When membranes were added, a protease-protected form of the hybrid appeared. This hybrid was also glycosylated as deduced from
Fig. 3 (lanes 1–6) shows the results of analyses in which we have tested whether the p62 signal sequence region confers stable membrane attachment to the p62-dhfr hybrid. Microsome-supplemented translations were adjusted to pH 11.1–11.5 with NaOH, incubated on ice for 10 min, and then separated into a membrane pellet and supernatant fraction by ultracentrifugation. In all samples the p62-dhfr polyptides were isolated using an anti-dhfr antibody. SDS-PAGE shows that the hybrid protein segregates almost quantitatively into the supernatant fraction (compare lane 1 with lane 2).

In similar conditions an integral membrane protein, the human transferrin receptor, was found to sediment with the membranes into the pellet fraction and a secretory protein, Ig light chain, was only recovered in the supernatant (not shown). If the acceptor peptide for glycosylation was included in the in vitro translation and the mixture then analyzed we found that the now unglycosylated but still translocated p62-dhfr hybrids were again mostly found in the supernatant fraction (lanes 3 and 4). Lanes 5 and 6 show the analyses with the control peptide.

To see whether the C protein exerts an influence on the translation phenotype of the p62-dhfr protein the p62-dhfr plasmid (see Fig. 1), lacking the C gene, was tested. The results shown in Fig. 4 clearly show that the p62-dhfr hybrid is translocated and glycosylated in the same way as when expressed from pCp62dhfr. Thus, apart from providing a free NH₂-terminal end to the p62-dhfr protein by autoproteolysis of the C-p62-dhfr precursor the C protein has no role in the translocation process.

We conclude that the 40 residue peptide from the p62 NH₂-terminal region confers a translocation positive phenotype to the p62-globin and p62-dhfr polyptides and therefore must contain a functional signal sequence. The translocated fusion proteins were also shown to be glycosylated. This must involve Asn₁³ of the p62 peptide as it is part of the only potential glycosylation site on the hybrid poly-peptides (Garoff et al., 1980; references on dhfr sequence in legend to Fig. 1). Finally, we can also conclude that the p62 signal sequence does not provide a stable membrane anchor to the translocated chain.

**Time Point of Asn₁³ Glycosylation during Polypeptide Chain Synthesis and Translocation**

To define at what time point during p62-dhfr chain synthesis the Asn₁³ becomes glycosylated we performed a time-course experiment essentially as described by Rothman and Lodish (1977) (Fig. 5). In this experiment a 150-μl translation was initiated. After 1.5 min ATA was added (0.075 mM) to block additional starting of chain synthesis. Then, at 0.5-min intervals, two 7.5 μl aliquots were removed; one for mixing with 40 μl of hot PAGE sample buffer (2% SDS) and the other one for further incubation after mixing with 0.75 μl of 20% TX-100. The first sample from each time point was used for the determination of the time needed for chain completion, which is a function of the translation rate, and the other one allowed determination of the time course of glycosylation of the translocated chain. Triton X-100 solubilizes the microsomal membranes and thereby inactivates glycosylation (but not chain elongation). Therefore, only those p62-dhfr chains that have presented Asn₁³ to the glycosylation machinery before TX-100 addition have had the possibility to become glycosylated. In Fig. 5, lanes 1–10, one can see that completed p62dhfr chains (197 residues with initiator Met) appear after a 3-min incubation from the time point of ATA addition.

[Figure 5. Time course of p62 dhfr glycosylation. A 150-μl translation was initiated. After a preincubation time of 1.5 min ATA was added to inhibit further initiation of chain synthesis. Then, at intervals of 0.5 min (indicated by 0.5', 1.0', 1.5', 2.0', 2.5', 3.0', 3.5', 4.0', 4.5', and 5.0') two 7.5-μl samples were removed, one for mixing with PAGE sample buffer and another one for mixing with TX-100 (final concentration 1%) and further incubation at 30°C (for a total time of 20 min after ATA addition as indicated by the lower row of time points in the figure). Lanes 1–10 show the samples removed for mixing with the PAGE sample buffer. From these results the approximate rate of translation can be derived. Completed chains appear in the 3-min sample. Lanes 11–20 show the samples in which the membranes have been solubilized with Triton X-100 for inactivation of the glycosylation machinery. From these analyses it is possible to estimate when Asn₁³ is modified during p62-dhfr synthesis. The first glycosylated forms are clearly visible in the 1.5-min sample, a time point where only about half of the p62-dhfr chain has been synthesized. The nature of the material in the two weak bands seen in lanes 1–5 is unclear. Their transient appearance before the completion of the p62-dhfr chain suggests that they represent complexes of nascent p62-dhfr chains.]
The p62 signal sequence has catalyzed the insertion of about half the molecules, in contrast to the noninserted molecules which have an apparent mass of about 52 kD. The glycosylated and translocated nature of the 60-58 kD material was clearly demonstrated in experiments similar to those described above for the p62-dhfr hybrid molecule (not shown).

1.5-min preincubation without ATA then the total time for chain synthesis is ~3.75 min (3 + 0.75 min). This corresponds to a mean translation rate of 52.5 peptide bonds per min. Lanes 11-20 show that glycosylated chains appear in all those samples that have had the membranes intact for 1.5 min or more after ATA addition. This means that p62-dhfr chains that have been elongated for ~2.25 min (1.5 min incubation after and 0.75 min before ATA addition), to the length of ~118 residues already carry a sugar unit at Asn13. As ~60 residues of the nascent chain are required to span the ribosome and the lipid bilayer, we conclude that glycosylation occurs when the first 50-60 residues of p62-dhfr appear within the lumen of the ER (Malkin and Rich, 1967; Blobel and Sabatini, 1970; Bergman and Kuehl, 1977; Smith et al., 1978; Glabe et al., 1980; Randall, 1983).

We also studied the timing of the glycosylation of Asn3 in its normal background, i.e., during p62 chain synthesis. For this experiment we used the pGEM SFVd-4 construct. This encodes the C and the p62 membrane protein variant, p62-d-4, in which a few residues of the cytoplasmic protein domain have been exchanged as compared to the wild type sequence (see Materials and Methods and Fig. 1). Fig. 6, lane 2, shows that RNA, which has been transcribed from this construct, directs the synthesis of C and p62 d-4 chains. The protein has catalyzed correct C-p62 cleavage and the p62 signal sequence has catalyzed the insertion of about half of the p62 d-4 chains across the added microsomal membranes. These migrate as glycosylated 60-58 kD proteins in contrast to the noninserted molecules which have an apparent molecular mass of ~52 kD. The glycosylated and translocated nature of the 60-58 kD material was clearly demonstrated in experiments similar to those described above for the p62-dhfr hybrid molecule (not shown).

 Altogether there are four glycosylation sites within the p62 d-4 sequence. These correspond to Asn residues at positions 13, 60, 266, and 328 (see Fig. 1). Fig. 6 (lanes 3-16) shows the time course of the four glycosylation events during C-p62 d-4 translation. A slightly different protocol was followed in this experiment as compared to that with p62-dhfr. Seven translations were initiated in parallel and after a 3-min incubation these were put on ice and ATA was added. Elongation of the already initiated chains was then continued for a total of 35 min, however, so that Triton X-100 was added to individual samples at 5, 10, 15, 20, 25, 30, and 35 min. At these time points half of each sample was also removed and translation stopped by cooling on ice. Lanes 3-9 show the SDS-PAGE of the samples that had received Triton X-100 at different time points. We found the sequential appearance of p62 d-4 polypeptides with no carbohydrate (lane 3), with one and two units added (seen as two new bands with slower migration in lanes 4 and 5), with three units (lane 6), and all four sugar units (lanes 7, 8, and 9) attached to the protein backbone as the translation proceeded coordinately with time. Note that the four glycosylation events result in different degrees of increase of the size of p62 d-4. The second event causes the largest increase and the third one the smallest. As the sugar unit added at each step should be the same we think that these differences reflect some conformational changes in the p62 folding which occur coordinately with glycosylation.

In lanes 10-16 we have analyzed the samples that were withdrawn at the different times but were kept on ice. As expected, we see a sequential appearance of the capsid...
protein (in the 10-min sample) and then the p62 d-4 protein (barely visible in the 20-min sample). The p62 d-4 protein is partly present in its glycosylated and partly in its unglycosylated form. Using 21.5 min as a rough estimate for the translation time of the 746 residue long C-p62 d-4 chain (time point of p62 d-4 detection, 20 min, plus half of the 3-min preincubation time without ATX) we have calculated the translation rate and derived the approximate earliest time points when the four glycosylation sites of p62 d-4 should be available for modification. According to these, Asn13 and Asn90 should be the only sites available for glycosylation in the 10-min sample, shown in lane 4, and the most abundant ones presented for modification in the 15-min sample, shown in lane 5. Therefore, it appears reasonable to assume that those chains of these two samples which have obtained two sugar units carry these on the aforementioned two sites. Thus, the peptide region with Asn13 seems to be target for rapid modification also when present in its normal background, that is with the p62 protein.

Discussion

The fact that the 40 residue fragment of the NH2-terminal region of the p62 protein is able to translocate two different reporter molecules into microsomes constitutes in our mind convincing evidence for signal sequence activity in this protein fragment. A more precise location of the p62 signal sequence within the 40 residue p62 fragment can be done with the aid of the known consensus features of a signal sequence. The most typical characteristic of a signal sequence is a stretch of 10-12 uncharged residues, mostly hydrophobic ones (von Heijne, 1985). This part of the signal sequence probably forms an alpha helix in the ER membrane (Emr and Silhavy, 1983; Briggs et al., 1985, 1986; Kendall et al., 1988). The only possible candidate region within the 40 residue p62 fragment having these features is the 13 residue segment between Pro 3 and Pro 17 (see box in uppermost sequence in Fig. 7). The Pro-rich region in the middle of the 40 residue fragment would not form an alpha-helix, and the COOH-terminal part of the p62 segment contains a high number of charged residues. As shown in Fig. 7 these features are conserved in all those alphaviruses where the p62 protein has been sequenced. Thus, we find the experimental results, together with the structural considerations discussed above, highly indicative that the 16 NH2-terminal residues of p62 constitute its signal sequence.

Eventually, the signal sequence of the p62 protein becomes translocated across the membrane of the ER into its luminal space. In here it is found as a glycosylated peptide which is part of a 66 amino acid residues long "pro-piece of the p62 protein. This pro-peptide, called E3, is cleaved at a late stage during virus assembly (de Curtis and Simons, 1988) and is then either released into the extracellular medium as a soluble protein (Sindbis virus) or remains as a peripheral protein subunit on the virus spike (SFV) (Garoff et al., 1982; Mayne et al., 1984). Our present tests of the p62-globin and p62 dhfr hybrids in the high pH wash assay of membrane supplemented in vitro mixtures also support the notion that the p62 signal sequence does not remain bound to the membrane where it has exerted its function as a translocation signal.

In this work we like to use the glycosylation event at Asn13 of the signal sequence to mark the time point when the latter becomes released into the lumen of the ER. The crucial question then becomes whether it is reasonable to assume that the signal peptide has to be released from the ER membrane before it can become glycosylated. To answer this question we have to consider what is known about the topology of glycosylation as well as the way by which the p62 signal might interact with the ER membrane.

Today there is no exact information about how a signal sequence might be inserted into the ER membrane when exerting its function in chain translocation. However, the typical cytoplasmic orientation of the NH2-termini of membrane protein chains carrying a combined signal sequence-anchoring peptide suggests that signal sequences in general might direct their function in translocation through the insertion of their hydrophobic and uncharged stretch of amino acid residues into the membrane in such an orientation that the NH2-terminus of the signal remains on the outside of the ER mem-

![Figure 7. NH2-terminal amino acid sequences of p62 proteins of alpha viruses. The amino acid sequences of the 40 residue NH2-terminal peptide of the p62 protein of SFV (upper sequence) and the corresponding sequences of the p62 proteins of Ross River virus (RRV); Sindbis virus (Sin); Venezuelan Equine Encephalitis virus (VEE); and Eastern Equine Encephalitis virus (EEE) (Garoff et al., 1980; Rice and Strauss, 1981; Dalgarno et al., 1983; Kinney et al., 1986; Chang and Trent, 1987). Amino acid residues are given using the one letter code and they are numbered from the NH2-terminus. The boxes indicate that region in each sequence which best fulfills the consensus features of a signal sequence (the uncharged and hydrophobic region). The * symbols represent attachment sites for oligosaccharide and the (+) and (−) the presence of a charged amino acid side chain. Proline residues are labeled with a dot. The sequences are aligned according to maximum amino acid sequence homology.](image-url)
brane (Bos et al., 1984; Lipp and Dobberstein, 1986; Spiess and Lodish, 1986; Zerial et al., 1986; see also Shaw et al., 1988). In addition, it is known from physical studies using synthetic signal peptides and artificial lipid membranes that the signal peptides readily insert into the membrane and there obtain an alpha-helical conformation (Briggs et al., 1985, 1986; Batenburg et al., 1988; Cornell et al., 1989).

If the p62 signal sequence adapts such an orientation and conformation in the ER membrane it would mean that the glycosylation site at Asn13 would locate inside the membrane (von Heijne, 1985). In this location the site can hardly be accessible for the glycosylation machinery. (Note that in the related Ross River virus, the Venezuelan Equine Encephaliti
tis virus and Eastern Equine Encephalitis virus the corresponding glycosylation site is even closer towards the NH2-terminus, that is at Asn11, see Fig. 7.) According to several recent studies, glycosylation requires the exposure of the glycosylation site in the lumen of ER. Firstly, it has been shown that the binding protein for the glycosylation site of N-linked oligosaccharides is a luminal 57-kD protein of the ER (Geetha-Habib et al., 1988). Secondly, one study with the asialoglycoprotein receptor and another one with the Coro
na virus E1 membrane protein demonstrate that luminaly oriented glycosylation sites are not used on transmembrane polypeptides if they locate very close to the membrane-binding segments of the chains (Mayer et al., 1988; Wessels and Spiess, 1988). In the case of the asialoglycoprotein receptor a site was not used if located 12 residues apart from the membrane anchor, however, if moved 8 more residues apart from the anchor it became glycosylated. In the case of the Corona virus protein a site just adjacent to the combined signal sequence-anchor peptide remained unglycosylated, whereas an engineered site 24 residues further away was used for glycosylation. Such restrictions in glycosylation are most likely to be explained by sterical problems in attaching the very spacious sugar unit (Lee et al., 1984; see also Wier and Edidin, 1988) onto acceptor sites that are fixed in a position which is close to the membrane plane.

Therefore, we assume that the p62 signal sequence, with its glycosylation site at Asn9, cannot become glycosylated before it has been released into ER lumen. As this glycosyla
tion event was shown to occur at an early stage of chain translocation it follows that this signal sequence can only in
teract with the ER membrane during the beginning of chain translocation. In other words, the signal sequence of p62 can only function at the initiation stage of chain translocation and has no role in completing this transfer process. If the latter would be true we would have expected that the signal sequence glycosylation would have occurred first after all of the luminal domain of the p62 d-4 chain would have been translated and translocated.

The importance of our results in this work lies in the fact that they rule out translocation models in which the signal sequence would have a role throughout the whole process of chain translocation. For instance, if the translocation site is represented by a multisubunit protein complex forming an aqueous channel across the membrane for chain transfer (see signal hypothesis, Blobel and Dobberstein, 1975; amphipatic tunnel hypothesis, Rapoport, 1985; translocator protein hypothesis, Singer et al., 1987a,b), then the signal sequence could be involved in its assembly or "opening" but apparently not for keeping it together or open until chain transfer is completed (as suggested in Singer et al., 1987a).

Similarly, when considering models in which the chain transfer occurs directly through a lipid membrane (see the helical hairpin hypothesis, Engelmann and Steitz, 1981; direct transfer model, von Heijne and Blomberg, 1979; phospholipid channel hypothesis, Nesmayanova, 1982) the interaction of the signal sequence with the lipid bilayer could be of importance only at the stage of translocation initiation but not at the actual chain transfer step.

The possibility that our results about p62 protein translo
cation would be unique to the viral system and different from the general translocation process in the ER we find most unlikely. Several results from this and earlier works suggest that the signal sequence of the p62 protein functions much in the same way as cleavable ones do. Firstly, studies with a temperature-sensitive mutant of SFV, ts3, have shown that the signal sequence of p62 requires a free NH2-terminal end for function (Hashimoto et al., 1981). At the nonpermissive temperature the ts3 mutant is defective in cleavage be
tween the C and the p62 protein region of the protein precurs
or because of a mutation that inactivates the autoproteolytic activity of C. This defect results in a translocation negative phenotype for the p62 protein. Secondly, the p62 signal se
quence has been shown to be SRP dependent. If the mRNA for the structural proteins of SFV is translated in vitro in a wheat germ-derived system that is supplemented with salt-washed (and SRP-deprived) membranes then p62 transloca
tion is observed only in the presence of exogenous SRP (Bonatti et al., 1984). If SRP is supplemented without membranes then p62 translation is arrested. Thirdly, our time course study about p62 synthesis and glycosylation in this work clearly demonstrates that the p62 chain is translocated cotranslationally across the ER membrane. This was also suggested by earlier studies in vitro (Garoff et al., 1978; Bonatti et al., 1984). In these studies it was shown that both microsomal membranes as well as SRP have to be added to the synthesis mixture before extensive lengths (~100 amino acid residues) of the p62 chains have been translated.

It is also possible to speculate on a mechanism in which the p62 signal sequence would be released from a putative translocation site by being replaced by another signal sequence-like structure in the p62 polypeptide. However, such a "rescue" mechanism appears improbable as the p62 signal sequence was found to be glycosylated early during translation of both the p62 polypeptide as well as the signal sequence-dhfr hybrid chain.

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