A Nascent Secretory Protein May Traverse the Ribosome/Endoplasmic Reticulum Translocase Complex as an Extended Chain*

(Received for publication, November 20, 1995, and in revised form, January 9, 1996)

Paul Whitley, IngMarie Nilsson, and Gunnar von Heijne†
From the Department of Biochemistry, Stockholm University, S-106 91 Stockholm, Sweden

We have measured the minimum number of residues in a translocating polypeptide required to bridge the distance between the P-site in endoplasmic reticulum-bound ribosomes and the luminaly disposed active site of the oligosaccharyl transferase. The results suggest that a nascent chain may traverse the ribosome/translocase complex in a largely extended conformation, and that hydrophobic stop-transfer segments have a more compact, possibly α-helical conformation in the translocase.

Protein translocation through the membrane of the endoplasmic reticulum (ER) is catalyzed by a complex multisubunit translocation machinery comprising cytoplasmic, integral membrane, and luminal components (1, 2). It has been proposed that the integral membrane components form a water-accessible channel in the membrane through which nascent chains can pass (3). Experimental evidence based on a wide range of methods such as electrophysiology (4), urea extraction (5), photo-cross-linking (6), and fluorescence quenching (7, 8) all support this proposal. Although the environment of nascent translocating polypeptide chains has thus been quite well characterized, their conformation during passage through the ER translocase is not known. In mitochondria, it has been shown that nascent chains traverse the two mitochondrial membranes in a largely extended conformation (9).

Previously, we have found that the luminaly oriented active site of the oligosaccharyl transferase (OST) enzyme (itself part of the translocation complex; Ref. 10), is positioned at a well defined distance above the surface of the ER membrane and can thus be used as a fixed point of reference against which the location of various parts of a nascent polypeptide in the translocase can be determined (11, 12). We now report measurements of the minimum length of polypeptide chain required to bridge the distance between the ribosomal peptidyl transferase site (P-site) on the cytoplasmic side of the ER membrane and the OST active site on the luminal side. The minimum distance has been measured both for nascent chains corresponding to a globular protein domain that normally becomes fully translocated to the lumen of the ER and for nascent chains containing a hydrophobic stop-transfer sequence which interrupts translocation and ultimately becomes integrated into the lipid bilayer. Our results suggest that non-hydrophobic nascent chains may adopt a fully extended conformation during passage through the ribosome/translocase complex, whereas the hydrophobic stop transfer sequence appears to form a more compact, possibly helical, structure when located in the translocase.

MATERIALS AND METHODS

Enzymes and Chemicals—Unless otherwise stated, all enzymes as well as plasmid pGEM1 and wheat germ lysate were from Promega Biotech (Madison, WI). SP6 DNA polymerase, ribonucleotides, deoxyribo- nucleotides, dideoxyribonucleotides, and the cap analog m7G(5’)ppp(5’)G were from Pharmacia Fine Chemicals (Uppsala, Sweden). Proteinase K was from Merck Sharpe and Dohme. [35S]Methionine was from Amersham Corp. Puromycin was from Sigma. Oligonucleotides were purchased from Kebio Lab (Stockholm, Sweden).

DNA Techniques—Insertion of the stop transfer sequence QQL17VKKKK into the P2 domain of Lep was performed by introducing BclI and Ndel restriction sites in codons 214 and 220, respectively. Double-stranded oligonucleotides coding for the above amino acid sequence were then cloned between the BclI and Ndel restriction sites. Site-specific mutagenesis used to introduce an Asn Ser Thr glycosylation acceptor site in position 200 of Lep and to introduce restriction enzyme cleavage sites was performed according to the method of Kunkel (13), as modified by Geisselsoeder et al. (14). For cloning and expression from the pGEM1 plasmid, the 5’ end of the lep gene was modified, first, by the introduction of an XbaI site, and second, by changing the context 5’ to the initiator ATG codon to a “Kozak consensus” sequence (15). Thus the 5’ region of the gene was modified to: ...ATAACCCCTCTAGAGCCACCATGGCGAATATG... (XbaI site and initiator codon underlined). Mutants of Lep made in phage M13 were cloned into pGEM1 behind the SP6 promoter as an XbaI-Smal fragment. All constructs in pGEM1 were confirmed by sequencing of the plasmid DNA.

Templates for in vitro transcription of truncated mRNA were prepared using the polymerase chain reaction (PCR) to amplify fragments from pGEM1 plasmids containing the desired DNA constructs. The 5’ primer was the same for all PCR reactions and had the sequence 5’-TTCGCTCAACAAACCGACTC-3’. This primer is situated 210 bases upstream of the translational start, and all amplified fragments thus contained the SP6 transcriptional promoter from pGEM1. The 3’ primers were chosen according to the positions of the truncations and were designed to have approximately the same annealing temperature as the 5’ primer. None of the 3’ primers contained translational stop codons. Amplification was performed with a total of 30 cycles using an annealing temperature of 59°C. The amplified DNA products were separated on a low melting-point agarose gel, excised, and purified using Wizard PCR purification resin (Promega) as described in the manufacturers protocol.

In Vitro Transcription—Truncated mRNAs were transcribed from the SP6 promoter. The reaction mixture was as follows: 10 μl of 5× Promega transcription buffer, 5 μl of 100 m Mug diithiorethiol, 2 μl of 25 mM ribonucleotide triphosphates (A, U, C, and G), 5 μl of 5 mM m7G(5)ppp(5)IG, 3.5 μl of RNA oligo, 20 μl of DNA template, 2.5 μl (50 units) of RNasekase, 2 μl (40 units) of SP6 RNA polymerase. Transcriptions were carried out at 37°C for 1 h. The reactions were stopped by extracting with an equal volume of phenol/chloroform (1:1) followed by extraction with chloroform. Samples were precipitated by the addition of NaCl to 50 mM and 2 volumes of ethanol. The precipitates were pelleted by centrifugation for 10 min in a microcentrifuge at 4°C. The pellets were washed in 70% and then 95% ethanol and dried. The pellets were
redissolved in 100 μl of H2O, and 5 μl were run on a 1% agarose gel to verify that the RNA band was at least 5–10 times more intense than the DNA template band.

In Vitro Translation—In vitro translation of [35S]methionine-labeled proteins from the in vitro synthesized mRNA were carried out in 50-μl reactions using wheat germ extract according to instructions supplied by the manufacturer. Translation reactions were performed at 25°C for 1 h. When relevant, the translation mixes were supplemented with 1 μl (2 A260 equivalents) of EDTA-washed dog pancreas microsomes (16, 17) and 1 μl of canine SRP (20 μm final concentration) prepared as described (18). For puromycin-treated samples, translation in the presence of microsomes and SRP was performed as above, followed by the addition of 2 μl of 30 mM puromycin and incubation at 25°C for another 10 min. At the end of the reaction, samples were acid precipitated by the addition of an equal volume of 20% trichloroacetic acid and then processed for immunoprecipitation with a polyclonal antiserum against Lep. Gels were visualized on a Fuji BAS1000 phosphorimager and quantitated using the MacBas 2.1 software.

Translation in reticulocyte lysate in the presence of dog pancreas microsomes was performed as described (19). Translocation of polypeptides to the luminal side of the microsomes was assayed by resistance to exogenously added proteinase K and by prevention of N-linked glycosylation through competitive inhibition by addition of a glycosylation acceptor tripeptide (N-benzoyl-Asn-Leu-Thr-N-methylamide) as described (11).

RESULTS

65 Residues Are Required to Bridge the Distance between the Ribosomal P-site and the OST Active Site—A well characterized model protein (Lep) (Fig. 1), normally located in the inner membrane of Escherichia coli, was used to measure the number of residues required to bridge the distance between the P-site and the OST active site (the “minimum glycosylation length”). When expressed in vitro in the presence of dog pancreas microsomes, Lep inserts in an SRP-dependent manner with the same orientation as in E. coli, i.e. with both the N-terminal tail and the C-terminal P2 domain in the lumen (Refs. 11 and 20; data not shown). Lep is efficiently glycosylated on Asn214 when expressed as a full-length protein in the ER (Refs. 11 and 20; data not shown). Lep is efficiently glycosylated on Asn200 (Fig. 3A, lane 2), truncation 61 residues away; lanes 4–6, truncation 56 residues away; lanes 7–9, truncation 51 residues away; lanes 10–12, truncation 61 residues away; lanes 13–15, truncation 63 residues away; lanes 16–18, truncation 64 residues away; lanes 19–21, truncation 65 residues away; lanes 22–24, truncation 66 residues away. Band a, non-glycosylated Lep; band b: glycosylated Lep.

To measure the minimum glycosylation length, mRNAs truncated in the P2 domain of Lep at various defined positions C-terminal to the potential glycosylation acceptor site (Asn214, Ser-Thr) were generated by PCR and in vitro transcription. When translated in a wheat germ lysate expression system in the presence of SRP, the resulting truncated proteins (which do not contain a translational stop codon) remain bound to the ribosomal P-site at their C-terminal end (21), while the more N-terminal parts of the P2 domain extend through the ribosome/translocone complex into the lumen of the ER.

As shown in Fig. 2 (panel B, lanes 1–3), when the truncation was 39 amino acid residues C-terminal to the acceptor site, no glycosylation was seen unless the nascent chain was released from the ribosome by treatment with puromycin, indicating that the protein had been correctly targeted to the ER membrane but did not extended sufficiently far from the P-site for the potential glycosylation site to reach the OST active site. Similar results were obtained for nascent chains truncated 51, 56, 61, 63, and 64 residues away from the potential glycosylation acceptor site (lanes 4–18). Finally, for truncations 65 and 66 residues away from the potential glycosylation acceptor site, a sharp increase in the amount of glycosylation in the absence of puromycin was observed (lanes 19–24). We conclude that a minimum of 65 amino acid residues is required to span the distance between the ribosomal P-site and the OST active site.

The Presence of a Stop-transfer Sequence Increases the Minimum Glycosylation Length—Based on the results of a previous study where the number of residues between the luminal end of a hydrophobic transmembrane segment and the OST active site was measured, we suggested that transmembrane segments may be lipid-exposed and have a helical conformation when located in the ER translocone (12). Since the results reported above indicated that a nascent chain not containing any extensive hydrophobic stretches may traverse the translocone in an extended conformation (see “Discussion”), we considered the possibility that the minimum glycosylation length may be different if a hydrophobic segment is present in the nascent chain between the glycosylation acceptor site and the C terminus.

We thus constructed a protein, Lep-ST (Fig. 3A), that contains an engineered glycosylation site Asn200-Ser-Thr replacing amino acids 200–202 and the stop-transfer sequence QQQL17VKKKK inserted between amino acid residues 215 and 220. When expressed in the presence of microsomes, Lep-ST was efficiently glycosylated on Asn200 (Fig. 3B, lane 2) and...
glycosylation was inhibited by inclusion of a glycosylation acceptor peptide (N-benzoyl-Asn-Leu-Thr-N-methylamide) in the reaction mixture (lane 3). A short fragment representing the glycosylated loop between the second transmembrane segment and the hydrophobic stop-transfer sequence was protected from proteinase K digestion (lanes 4 and 5), demonstrating that the topology of Lep-ST was as depicted in Fig. 3A.

Truncations were made at different sites downstream of the stop-transfer segment, and glycosylation in the presence of microsomes was assayed as above. As shown in Fig. 4, for truncations 52, 66, and 69 residues away from the potential glycosylation acceptor site no glycosylation was seen in the absence of puromycin (lanes 1–9). For truncations 70 and 71 residues away from the potential glycosylation site, there was a sharp increase in the amount of glycosylation observed in the absence of puromycin (lanes 10–15), and complete glycosylation was observed when the truncation was 74 and 91 residues away (lanes 16–21). It thus appears that the presence of a long hydrophobic stretch makes the nascent chain spanning the translocase complex more compact.

DISCUSSION

By measuring the minimum number of residues required to bridge the distance between the ribosomal P-site and the OST active site, we have sought to indirectly determine the conformation of a nascent chain in transit through the ER membrane. Our data show that a translocating nascent chain corresponding to a globular domain of a protein needs to be extended by a minimum of 65 amino acid residues from the P-site in the ribosome to be able to interact with the OST active site (Fig. 5). This length is increased to 71 residues when a 18-residue-long hydrophobic stop-transfer sequence is present in the nascent chain.

These results are broadly consistent with previously reported data, but have a significantly higher precision. An early study provided a rough estimate of the P-site/OST distance of 45–95 residues (22). More recently, it has been shown that...
protease digestion of nascent secretory polypeptides in detergent permeabilized microsomes results in protected fragments of around 70 amino acid residues (23), and that photochemical cross-linking to Sec61 is possible from position 30 (6). From the minimum length of a nascent chain required for processing by signal peptidase (SPhase), one can estimate that roughly 100 residues are needed to span the distance between the signal peptide active site and the P-site in the ribosome (6, 30, 31), implying that the OST and signal peptide active sites are not immediately adjacent in the complex.

We have shown previously that at least 15 spacer amino acid residues are needed after the end of the hydrophobic core of a nascent chain when it reaches a length of 50–60 residues (24). Since these physical dimensions are only rough estimates, we cannot formally exclude that a short segment of a nascent chain is in fact helical, although we consider this unlikely.

In agreement with the suggestion of an extended conformation of the nascent chain, we find that the introduction of an 18-residue-long hydrophobic stop-transfer segment between the glycosylation acceptor site and the end of the nascent chain leads to an increase in the minimum glycosylation length of 6 residues, suggesting a more compact conformation of the nascent chain. Since the hydrophobic segment is located 19 residues downstream of the glycosylation acceptor site and since the acceptor site must be at least 17 residues away from the hydrophobic segment for glycosylation to be possible in this construct, the stop-transfer segment is located within the translocase complex in the critical constructs (truncations 69–74 residues away from the acceptor site). In the translocase, stop-transfer segments are believed to be in a partially lipid-exposed environment (12, 29) and thus most likely in a helical conformation. Indeed, if 18 residues in a nascent chain were forced from a fully extended to an α-helical conformation, 10 extra residues would need to be added to the chain in order to bridge a defined end-to-end distance. The 6-residue shift in the minimum glycosylation length that we observe is thus in reasonable agreement with an extended conformation for polar nascent chains and a helical conformation for hydrophobic segments.

In summary, a model where the nascent polypeptide chains pass through a water-accessible translocase channel in a largely extended conformation is consistent with all the available data. Further, hydrophobic stop-transfer sequences appear to have a more compact, possibly helical conformation when located within the translocase, suggesting a more lipid-exposed environment.

Acknowledgments—Dog pancreas microsomes were provided by Dr. M. Sakaguchi (Fukuoka, Japan). Glycosylation acceptor tripeptide was provided by Dr. H. Garoff (Huddinge, Sweden). Canine SRP was provided by Dr. T. Rapoport (Cambridge, MA).

REFERENCES

1. Rapoport, T. A. (1992) Science 258, 931–936
2. Brodsky, J. L., and Schekman, R. (1993) J. Cell Biol. 123, 1353–1365
3. Blobel, G., and Dobberstein, B. (1975) J. Cell Biol. 67, 835–851
4. Simon, S. M., and Blobel, G. (1991) Cell 65, 371–380
5. Gilmore, R., and Blobel, G. (1983) Cell 42, 497–505
6. Mothes, W., Prehn, S., and Rapoport, T. A. (1994) EMBO J. 13, 3937–3982
7. Crowley, K. S., and Johnson, A. E. (1993) Cell 73, 1101–1115
8. Walter, P., and Blobel, G. (1983) Methods Enzymol. 96, 682–691
9. Ungermann, C., Neupert, W., and Cyr, D. M. (1994) Science 260, 1250–1253
10. Gérlich, D., and Rapoport, T. A. (1993) Cell 75, 615–630
11. Nilsson, I., and von Heijne, G. (1993) J. Biol. Chem. 268, 5798–5801
12. Nilsson, I., Whitley, P., and von Heijne, G. (1994) J. Cell Biol. 126, 1127–1132
13. Kunkel, T. A. (1987) Methods Enzymol. 154, 367–382
14. Gerst, P. W., and Unwin, P. (1987) Biotechniques 5, 786–791
15. Kazak, M. (1989) Mol. Cell. Biol. 9, 5073–5080
16. Sakaguchi, M., Tomiyoshi, R., Kurokawa, T., Mihara, K., and Omura, T. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 16–19
17. Walter, P., and Blobel, G. (1983) Methods Enzymol. 96, 84–93
18. Walter, P., and Blobel, G. (1983) Methods Enzymol. 96, 682–691
19. Liljestrom, P., and Garoff, H. (1991) J. Viral. 65, 147–154
20. Johansson, M., Nilsson, I., and von Heijne, G. (1993) Mol. & Gen. Genet. 239, 251–256
21. Gilmore, R., Collins, P., Johnson, J., Kellaris, K., and Rapiejko, P. (1991) Methods Cell Biol. 34, 223–239
22. Glabe, C. G., Hanover, J. A., and Lennarz, W. J. (1980) J. Biol. Chem. 255, 9236–9242
23. Matlack, K. E. S., and Walter, P. (1995) J. Biol. Chem. 270, 6170–6180
24. Bergman, L., and Kuehl, W. (1979) J. Biol. Chem. 254, 8869–8876
25. Wang, S., Sakai, H., and Wiedmann, M. (1995) J. Cell Biol. 130, 519–528
26. Milligan, R., and Unwin, P. (1986) Nature 321, 693–695
27. Bologa, M., and Splinter, E. (1991) J. Mol. Biol. 55, 1471–1474
28. Yonath, A., and Wittmann, H. (1989) Trends Biochem. Sci. 14, 329–335
29. Martoglio, B., Hofmann, M. W., Brunner, J., and Dobberstein, B. (1995) Cell 81, 207–214
30. Kurokawa, T., Sakaguchi, M., Mihara, K., and Omura, T. (1993) J. Biochem. (Tokyo) 114, 541–546
31. Nicchitta, C. V., Murphy, E. C., Haynes, R., and Shelnitz, G. S. (1995) J. Cell Biol. 129, 957–970

2 I. Nilsson, H. Andersson, P. Whitley, G. von Heijne, manuscript in preparation.
A Nascent Secretory Protein 5 Traverse the Ribosome/Endoplasmic Reticulum Translocase Complex as an Extended Chain
Paul Whitley, IngMarie Nilsson and Gunnar von Heijne

J. Biol. Chem. 1996, 271:6241-6244.
doi: 10.1074/jbc.271.11.6241

Access the most updated version of this article at http://www.jbc.org/content/271/11/6241

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 31 references, 15 of which can be accessed free at http://www.jbc.org/content/271/11/6241.full.html#ref-list-1