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Conformation and length of the signal sequence affect processing of secretory protein

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Processing of human lysozyme with artificially designed signal sequences was examined in an in vitro translation–translocation system and compared with their secretory capabilities in yeast. It has been shown that the conformation of the C-terminal region of the signal sequence and the length of the hydrophobic segment are important factors for efficient cleavage of the signal sequence.

Signal sequence; Human lysozyme; Secretion; Translocation

1. INTRODUCTION

At the initial step of membrane translocation of secretory proteins, signal sequences play a central role. We have designed simplified signal sequences with high secretory capability, using a yeast secretion system of human lysozyme (hLZM). As a result, the sequence of functional signals has been generalized as follows: Met-Arg-$(Leu)_n$-Pro(Xaa)-Ala-Leu-Gly where n equals 6–12 and Xaa is Leu, Ala, or Leu-Ala or can be omitted [1–3]. The secretory capability was dependent on the length of the hydrophobic segment (=$(Leu)_n$) and was maximal at n=8 [1].

On the basis of the results obtained in yeast, we have chemically synthesized artificial signal peptides and analyzed their conformation by circular dichroism and ^1H nuclear magnetic resonance spectroscopy [4]. These studies indicated that a stable helical conformation in the hydrophobic segment is favorable but too stable a conformation in the C-terminal region is unfavorable for the function of the signal sequence.

However, it is still not clear which step in membrane transfer of a secretory protein (i.e. targeting, translocation of processing) requires the active conformation of the signal sequence, or which step is dependent on the length of the hydrophobic segment. In the present study, we analyzed both the protein translocation into canine pancreas microsomes and signal cleavages after the translocation using hLZM with artificial signal sequences.

2. MATERIALS AND METHODS

2.1. *Materials*

L-[^{35}S]Methionine (48 TBq/mmol) and rabbit reticulocyte lysate were purchased from Amersham Corp., Triton X-100 from Nacalai Tesque, m^{7}GpppG from Pharmacia LKB Biotechnology Inc., soybean trypsin inhibitor from Hoechst, and trypsin was from Sigma.

2.2. *Mutagenesis*

The expression plasmids of 88L6, 88L8PL, 88L8PG and 88L8PL2 were constructed by cassette mutagenesis. The small *Xba*I fragment of each expression vector of native hLZM with examined signal sequences [1,2] was replaced with that of 88Native [5].

2.3. *In vitro transcription*

mRNA encoding the sequence of signal-hLZM was obtained in vitro using a paired promoter SP6 system (Amersham). An *Xho*I–*Sma*I fragment carrying signal-hLZM coding region of each expression vector was replaced with a small *Sal*I–*Sma*I fragment of SP6 promoter vector pSPT18 (Boehringer). DNA fragments cloned into pSPT18 were transcribed with SP6 polymerase in the presence of 500 μM m^{7}GpppG.

2.4. *In vitro translation*

Translation reactions were carried out in a mixture containing 0.2 μg of mRNA, 16 μl of rabbit reticulocyte lysate, 110 mM KOAc, 1.5 mM $Mg(OAc)_2$, and 300 KBq of L-[^{35}S]methionine. The final volume was adjusted to 24 μl with water. Where indicated, 2 μl of canine pancreas microsomes prepared as described [6] was included. The mixture was incubated at 30°C for 60 min.

2.5. *Trypsin treatment of translation products*

The translation products were digested with trypsin (final concentration = 0.5 mg/ml) at 0°C for 30 min in the presence or absence of 1% Triton X-100. Proteolysis was stopped by addition of soybean trypsin inhibitor to a final concentration of 0.5 mg/ml. After addition of an equal volume of Laemmli's sample buffer [7], the samples were

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heated at 100°C for 5 min and analyzed by 15% SDS-PAGE. Autoradiography was performed using an imaging plate (Fuji Film) instead of X-ray film.

3. RESULTS

3.1. *In vitro translocation of hLZM*

Translocation of hLZM with several signal sequences (Table I) was analyzed in the cell-free system as shown in Fig. 1. The precursor of hLZM with the L8 signal sequence was translated in rabbit reticulocyte lysate from mRNA obtained by in vitro transcription (Fig. 1, lane 1). Inclusion of canine pancreas microsomes in the reaction solution resulted in the cleavage of the signal sequence and production of mature protein (Fig. 1, lane 2). After trypsin treatment, the precursor hLZM disappeared both in the absence and presence of Triton X-100, but the mature hLZM disappeared only in the presence of the detergent (Fig. 1, lanes 3 and 4). These results indicate that the processed hLZM was actually translocated across the membrane, but the unprocessed hLZM was not.

With designed signal sequences, L10, L12, L14 and L6, which were altered in the number of successive Leu residues in the hydrophobic segment, precursor hLZM was also processed, yet the amount of mature protein differed from each other (Fig. 1, lanes 6, 10, 14 and 18). Fig. 2 shows the proportion of processed protein as a function of the length of the hydrophobic region in the signal sequences. It shows that the most efficient signal

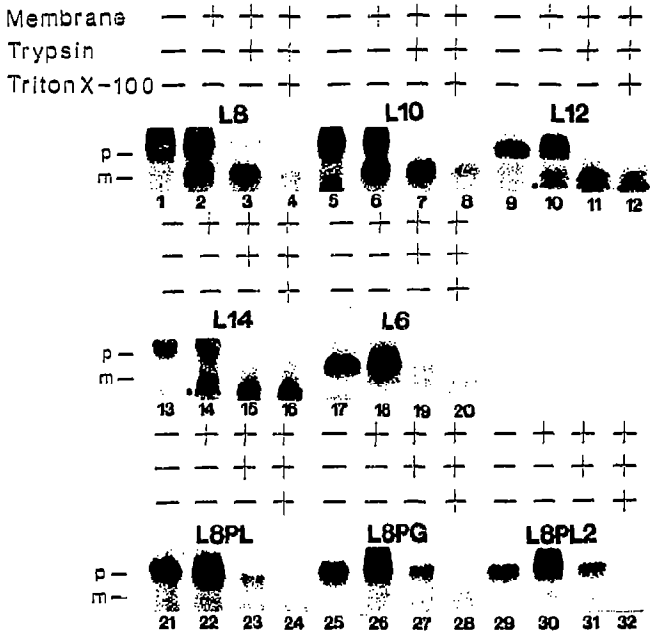

Fig. 1. In vitro translation, translocation and processing of hLZM. Lanes 1, 5, 9, 13, 17, 21, 25, and 29, and lanes 2, 6, 10, 14, 18, 22, 26, and 30 indicate translation of mRNA in the absence and presence of canine pancreas microsomes, respectively; lanes 3, 7, 11, 15, 19, 23, and 31, and lanes 4, 8, 12, 16, 20, 24, and 32, trypsin treatment of the samples (+microsomes) in the absence and presence of Triton X-100, respectively.

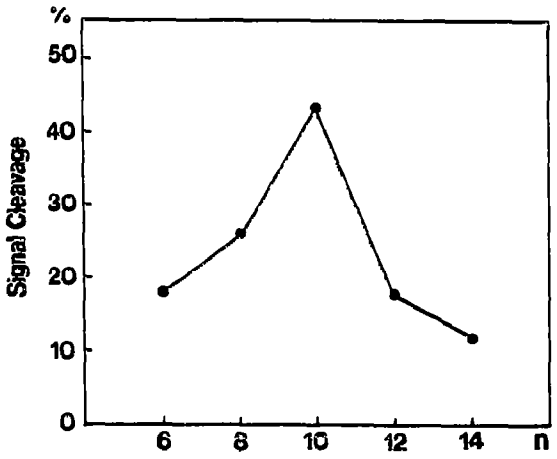

Fig. 2. Dependence of signal sequence cleavage on the length of the hydrophobic segment. *n* denotes the number of consecutive leucine residues in the hydrophobic segment of the signal sequences. The percentage of processed protein calculated from density measurement of each band on the gel (Fig. 1, lanes 2, 6, 10, 14, and 18) was regarded as the efficiency of signal cleavage. The sum of the density of mature and precursor proteins was taken as 100%. In the cases of L12 and L14, stable products which have smaller size than mature protein were included in the calculation as partially degraded precursor protein.

cleavage occurred with L10, and the efficiency reduced as the hydrophobic segment got longer or shorter.

It was found that some differences exist in the efficiency of signal cleavage between the cell-free system and in yeast. The L10 signal was cleaved more efficiently than the L8 signal in the cell-free system, though its secretory capability was slightly lower than that of L8 in yeast. Cleavage efficiency of the L12 signal was almost the same as L6, whereas secretion amount of hLZM with L12 in yeast was about one fourth of that with L6. After the translocation into the microsomes, the portion of L14 signal cleavage was nearly half that of L8 signal, whereas hLZM secretion with L14 was hardly detected in yeast (Table I).

The products which have lower molecular size than

Table I

Signal sequences studied

	Sequence	Secretion of human lysozyme (mg/l)
L8	MR $(L)_8$ PLAALG	5
L10	MR $(L)_{10}$ PLAALG	4.5
L12	MR $(L)_{12}$ PLAALG	0.5
L14	MR $(L)_{14}$ PLAALG	< 0.1
L6	MR $(L)_6$ PLAALG	2
L8PL	MR $(L)_8$ LLAALG	< 0.1
L8PG	MR $(L)_8$ GLAALG	0.4
L8PL2	MR $(L)_8$ PLLLAALG	0.1

The data for hLZM secretion are from our previous work [1,2].

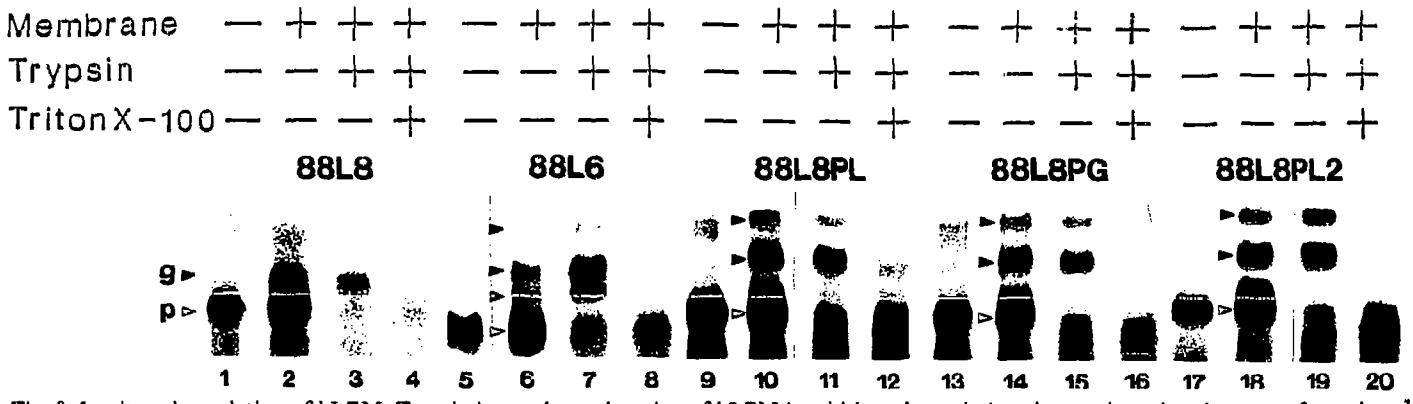

Fig. 3. In vitro glycosylation of hLZM. Translation and translocation of hLZM in which a glycosylation site was introduced were performed as described in Section 2.

that of mature hLZM were detected on the gel in the cases of L12 and L14 (Fig. 1, lanes 10, 11, 14, 15, indicated by dot). They are considered to be degraded forms of precursor proteins. They were resistant to trypsin even in the presence of the detergent (Fig. 1, lanes 12 and 16), suggesting that they may take some stable forms which occurred before the completion of protein translocation.

With L6 signal sequence, precursor protein was slightly resistant to trypsin treatment, though most of the other precursors were hardly detected (Fig. 1, lanes 3, 7, 11, 15, 19). It suggests that after the translocation, some of hLZM with L6 signal stays as a precursor.

The precursor proteins with another group of signal sequences, L8PL, L8PG and L8PL2, which were modified at the C-terminal region were synthesized as steadily as the others (Fig. 1, lanes 21, 25, and 29). However, their signal parts were not cleaved when the microsomes were included (Fig. 1, lanes 22, 26, and 30). These precursors were partially resistant to trypsin treatment (Fig. 1, lanes 23, 27 and 31). This indicates that the precursors with these signals were translocated across the microsome membrane, but were not processed to the mature form.

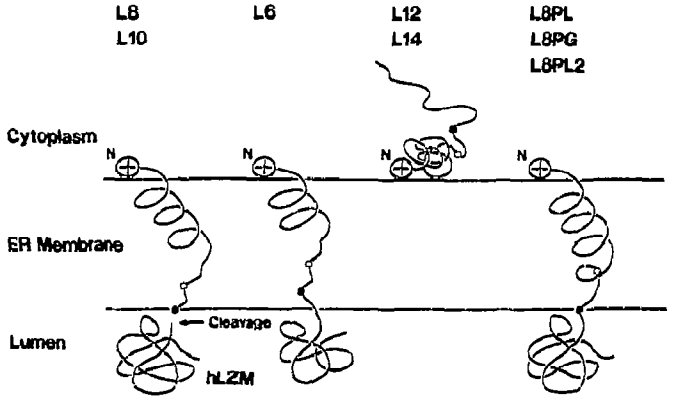

Fig. 4. Translocation and processing models of hLZM with mutant signal sequences. The residues at positions −3 and −1 are indicated by (○) and (●), respectively. See Section 4 for details.

3.2. *In vitro glycosylation of hLZM*

To verify that the translocation of these trypsin-resistant precursors did indeed take place, glycosylation of hLZM with L8, L6, L8PL, L8PG and L8PL2 signals was examined. An artificial glycosylation site, Asn^{88}-Ile^{89}-Thr^{90}, was introduced into hLZM as described [5]. The secretion capability of the L8 signal was not disturbed by glycosylation of hLZM in yeast (data not shown). Mutated precursors were named 88L8, 88L6, 88L8PL, 88L8PG and 88L8PL2, respectively.

The results of in vitro glycosylation are shown in Fig. 3. Synthesis of L8-hLZM precursor which has a glycosylation site (88L8) was similar to that of the native one (Fig. 1, lane 1 and Fig. 3, lane 1). The products synthesized in the presence of the microsomes contained a protein (Fig. 3, lane 2, g) with slower mobility than that of the precursor, and was resistant against trypsin (Fig. 3, lane 3). Treatment of the trypsin-resistant products with endo-*N*-acetylglucosaminidase H reduced the larger molecule and increased the unmodified one (data not shown). These results confirmed that 88L8 was translocated into the microsomes and was glycosylated.

88L6, 88L8PL, 88L8PG and 88L8PL2 were also glycosylated (Fig. 3, lanes 6, 7, 10, 11, 14, 15, 18 and 19), indicating that hLZMs with these signal sequences were actually translocated across the membrane. Three glycosylated forms of 88L6 and two glycosylated forms of the other mutants were detected on the gel. The lowest and the middle bands in lane 6 are considered to be glycosylated forms of mature and precursor proteins, respectively. This finding suggests that the L6 signal sequence was partially cleaved but some of the proteins translocated across the membrane were retained as a precursor. On the other hand, the lower bands in lanes

10 (L8PL), 14 (L8PG), and 18 (L8PL2) are glycosylated forms of the precursor, and no glycosylated form of mature protein was detected for these mutants. This indicates that these signal sequences were not cleaved after translocation. It is generally known that an *N*-linked oligosaccharide is processed in the endoplasmic reticulum with removal of glucose residues and of a particular mannose residue [8]. It is supposed that the top band in each lane is a glycosylated form in which oligosaccharide is processed uncompletely. In the case of hLZM with the L8 signal sequence, which can transport the protein efficiently, a single glycosylated form was detected (Fig. 3, lane 2). It suggests that incomplete processing of oligosaccharide may occur when the protein is not transported smoothly to the next organelle.

4. DISCUSSION

Based on these results, we propose models for the behavior of the precursor hLZMs with designed signal sequences during the translation–translocation steps (Fig. 4). L8 and L10 signal sequences have high secretory capability. They pass through the membrane after the translation, and expose the C-terminal residues to a lumen of the endoplasmic reticulum. They are cleaved by signal peptidase efficiently, and processed proteins go ahead to the next transport step. L6 signal sequence is considered to be not long enough to span the microsome membrane. It is assumed that the active site of signal peptidase is oriented to the lumen side of the microsome membrane, and it recognizes the residues at positions −3 and −1 of the signal sequence [9,10]. The cleavage site of the L6 signal is therefore not efficiently recognized by the signal peptidase, even though some portion of the precursor protein is translocated into the microsomes. This may explain the low activity of L6. L12 and L14 signal sequences may tend to aggregate on the membrane surface because of their high hydrophobicity. Most of them fail to enter the membrane though some portion of the signal sequence may succeed in extending through the membrane and being processed. hLZMs with aggregated signals must be partially degraded in the cytoplasm rapidly. It is supported by our previous results in vivo [3]. Pulse/chase experiments of hLZM with the L14 signal sequence showed that the precursor protein synthesized in yeast was spontaneously degraded. An alternative interpretation is that the precursor protein with L12 or L14 is retained in the membrane in the middle of the translocation. Recently, Tahara et al. have shown by electron cryomicroscopy that the signal L8 perturbs the membrane structure but L14 does not, and have suggested that the perturbation of the membrane structure is necessary for protein translocation [11]. According to these results, the trypsin-resistant form of L12- and L14-hLZMs may be a membrane-embedded translocation intermediate which was partially degraded in the cytoplasm. These intermediates may tightly associate with membrane, since they were not soluble even in the presence of Triton X-100. L8PL, L8PG and L8PL2 enter the microsomes and have hLZM translocated across the membrane as well as functional signal sequences. However, they are not cleaved by the signal peptidase after the translocation. This can be attributed to the conformational property of these signal sequences. We have recently shown that the C-terminal region of these signal sequences adopt a stable helical conformation, which is assumed to be unfavorable for the interaction with the signal peptidase, while functional signal sequences such as L8 can adopt an extended conformation, which seems to be more favorable for cleavage [4]. Our experimental results described here are consistent with these data.

A difference in cleavage efficiency of the signal sequences depending on the length of hydrophobic segments was found between yeast and the cell-free system (Table I and Fig. 2). It is perhaps a reflection of the species difference between these systems. A longer signal sequence segment may be required to stretch from the cytoplasm side to the lumen side in canine microsome membrane than in the yeast endoplasmic reticulum membrane. An excellent in vitro prokaryotic system for the translocation of secretory proteins has been established in *Escherichia coli*. Using the system with inverted membrane vesicles, detailed and quantitative studies on the charge and hydrophobicity of signal peptides were performed [12,13]. In vitro examinations using yeast microsomal membranes are needed to determine the precise features of these eukaryotic systems.

In conclusion, the following facts concerning structure and function of the signal sequence have been revealed. (i) For the targeting and translocation, the hydrophobic region of the signal sequence should be of suitable length to stretch across the membrane, and not be too long to prevent self-aggregation. (ii) After the translocation, the C-terminal region of the signal sequence should adopt a desirable conformation to be recognized by the signal peptidase. In addition, the length of the hydrophobic segment is also an important factor for this process.

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