Volume 238, number 2, 329–332 FEB 06384 October 1988

Alterations in the cleavage site of the signal sequence for the secretion of human lysozyme by *Saccharomyces cerevisiae*

Hitoshi Nagahora, Hirofumi Fujisawa and Yoshifumi Jigami

Bioorganic Chemistry Division, National Chemical Laboratory for Industry, Higashi 1-1, Tsukuba, Ibaraki 305, Japan

Received 27 July 1988

The amino acids corresponding to the cleavage site of a hybrid preprotein containing a chicken lysozyme signal and a mature portion of human lysozyme were altered. The processing of mutant signals of –3Pro and –3Asp/–1Ala decreased remarkably, while that of –2Pro was 75% of that of the native signal. The major cleavage site of –3Pro was the same as that of the native signal, but that of the –2Pro and –3Asp/–1Ala signals was shifted one residue closer to the N-terminal side than the original site. The cleavage of the –2Pro signal, which was identical to the native processing of pheasant prelysozyme, suggested that the signal peptidases in yeast and bird are similar.

Signal peptidase; Yeast; Cleavage site; Lysozyme signal; (Chicken)

1. INTRODUCTION

The signal peptide is composed of three structurally and functionally distinct regions: a basic N-terminal region, a central hydrophobic region, and a more polar C-terminal region defining the cleavage site. To confirm the cleavage specificity in yeast signal peptidase, we have engineered the cleavage site of a hybrid preprotein consisting of a CLY signal and a mature HLY, because the CLY signal contains common features described above and is functional (forms a correctly processed mature HLY) in yeast [1].

Von Heijne proposed the $(-3,-1)$ rules according to the statistical studies on signal sequences [2,3]; the residue at position -1 must be small and the residue at position -3 must not be aromatic, charged, or large and polar, and proline must be absent from positions -3 to $+1$ with some exceptions. In this paper we describe the correlation between the processing efficiency and the $(-3,-1)$ rules, and the shifts in the cleavage site of some mutant signals in yeast.

Correspondence address: Y. Jigami, National Chemical Laboratory for Industry, Higashi 1-1, Tsukuba, Ibaraki 305, Japan

Abbreviations: HLY, human lysozyme; CLY, chicken lysozyme; aa, amino acid; preHLY, precursor HLY

2. MATERIALS AND METHODS

2.1. *Materials*

Restriction enzymes and T_4 ligase were purchased from Toyobo or Takara Shuzo and used under the conditions as recommended by the suppliers. *Micrococcus lysodeikticus* cells and authentic HLY were obtained from Sigma. *Saccharomyces cerevisiae* KK4 (*α leu2 ura3 his1or3 trp1 gal80*) [4] was used as the host strain. Synthetic media [5], supplemented with an amino acid mixture (20–375 μg/ml) lacking leucine and with adenine sulfate (20 μg/ml) and uracil (20 μg/ml) [1] in addition to 2% glucose, were used to cultivate *S. cerevisiae*.

2.2. *DNAs*

The cassette mutagenesis methods [6] were used to construct a series of pFJ300 plasmids by replacing a part of the original DNA segments with double-stranded DNA oligomers which contain mutations at the residues in positions -1 and/or -3. The site-directed mutagenesis [7] was also performed to construct a series of pNJ300 plasmids by using the Amersham system. The multi-copy plasmid used to express HLY in yeast was the same as described in [1] except that the *GAL10* promoter was replaced with the cloned *ENO1* promoter [8].

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

2.3. *Assay of processing efficiency*

The efficiency of signal peptidase to cleave preHLY was evaluated either by the initial decrease in preHLY labeled with [^{35}S]methionine or by the ratio of mature HLY/(mature HLY + preHLY) measured by Western blotting. The lysozyme activity in the medium was assayed by monitoring the lysis of *M. lysodeikticus* cells at 450 nm.

2.4. *N-terminal sequence*

Secreted HLYs obtained from 2.5 l cultures were purified as described in [1] and were applied to an automatic protein sequencer (model 477A, Applied Biosystems). The aa sequences of the 5 N-terminal residues were quantitatively analysed by measuring the amounts of phenylthiohydantoin amino acids to determine the cleavage sites of mutant signals.

3. RESULTS

3.1. *Secretion of HLY*

The native CLY signal contains 18 aas like a yeast melibiase signal [9]. We replaced (i) Gly at position −1 with a larger aa (Ala, Ser, Thr or Val), (ii) Ala at position −3 with a rare locating aa (Val, Asp or Pro), (iii) Leu at position −2 with the β-turn forming aa (Pro). The effects of signal mutation on HLY secretion are summarized in table 1. The amounts of secreted HLY were almost the same in mutant signals containing −3Val, and were about half in mutant signals containing −1Ala, −1Ser, −3Val/−1Ala, and −2Pro, as those in the native signal. No lysozyme activity was detected in the other mutant signals without any concentrations of media under the assay conditions.

3.2. *Processing of mutant preHLY*

To evaluate the efficiency of preHLY processing, the conversion of preHLY containing mutations at position −1 to mature HLY was examined by immunoprecipitation. The amount of [^{35}S]methionine labeled preHLY decreased during the chase period in native and mutant signals, while that of mature HLY also decreased after a slight increase (fig.1A). As the processing was thought to be reflected better in the decrease of preHLY than in the increase of mature HLY, the decay curves of the mutant preHLYs were compared. It was found that the initial decreasing rate of preHLY decreased with the increase in the bulkiness of the aa at position −1 (fig.1B).

The correlation of the decay of preHLY

Table 1

Effect of mutant signal peptides on HLY secretion

Class[a]	Plasmid[a]	HLY activity[b] (units/ml)	Relative ratio	Processing[c] (M/M+P, %)	Relative ratio
WT	pFJ1053	64	1.00	68	1.00
I	pNJ301 (−1A)	31	0.48	68	0.85
	pNJ302 (−1S)	26	0.41	63	0.79
	pFJ342 (−1T)	0	0	41	0.51
	pFJ330 (−1V)	0	0	31	0.39
II	pFJ335 (−3V)	62	0.97	78	0.98
	pFJ371 (−3V/−1A)	31	0.48	78	0.98
	pFJ327 (−3V/−1V)	0	0	17	0.21
III	pFJ338 (−3V/−1D)	0	0	26	0.33
	pNJ303 (−1D)	0	0	6	0.08
	pFJ340 (−3D/−1A)	0	0	36	0.45
IV	pNJ305 (−2P)	26	0.41	60	0.75
	pNJ304 (−3P)	0	0	27	0.34

[a] WT, wild type preHLY; I, −1 mutants containing a different size of aa; II, −3 and/or −1 mutants containing a bulky aa; III, −3 and/or −1 mutants containing a charged aa; IV, −2 or −3 mutant containing a β-turn forming aa. aa are given as capital letters

[b] The lysis of 0.9 ml of *M. lysodeikticus* cells (0.15 mg/ml) was measured by adding 0.1 ml of culture supernatant after 4 days of cultivation. One unit of HLY is defined as the amount of enzyme which decreases 0.001 of A_{450} per min at 25°C. In this assay condition, the specific activity of an authentic HLY (purified from human milk, Sigma) was 130000 units/mg

[c] Determined by densitometric scanning of the autoradiogram from the Western blot gel and expressed as a percent of mature HLY/(mature HLY + preHLY)

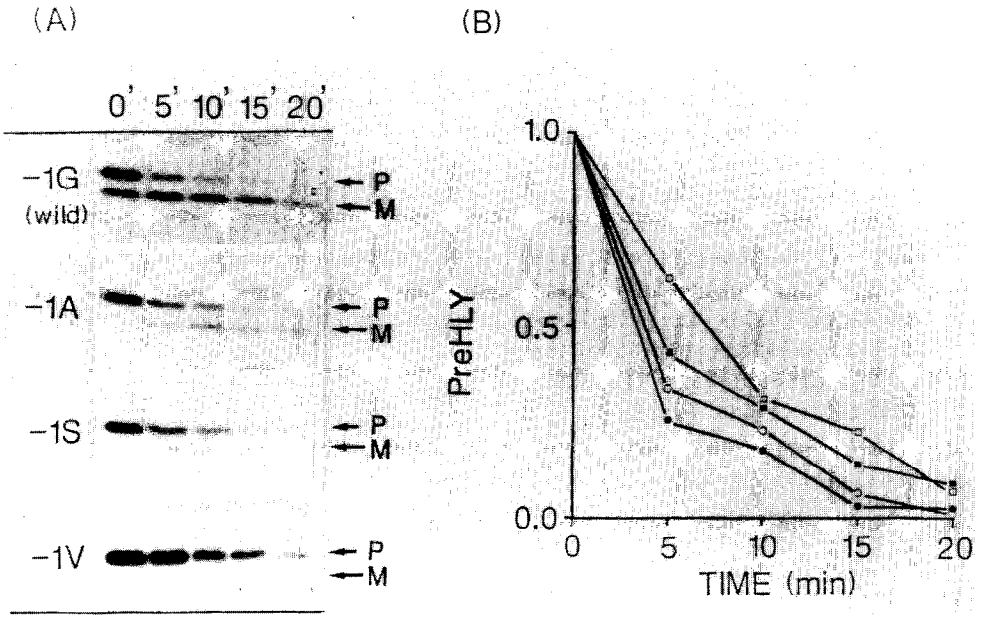

Fig.1. (A) Immunoprecipitation and SDS-polyacrylamide gel electrophoresis of mutant preHLYs. The KK4 cells transformed with the plasmid containing either wild type or mutant preHLY genes were spheroplasted by Zymolyase and labeled with [^{35}S]methionine (60 μCi) for 10 min. Immediately after initiation of the chase period unlabeled methionine (40 mM) and 50 mM cycloheximide were added. Aliquots were taken at various times and immunoprecipitated with HLY antiserum. Immunoprecipitates were analysed by autoradiography after gel electrophoresis. P, preHLY; M, mature HLY; −1G, −1A, −1S and −1V showed the signals containing Gly (wild type), Ala, Ser and Val at position −1, respectively. (B) Decrease of preHLYs during the pulse chase period. The intensity of the preHLY band (P) in A was measured by densitometry and the relative values at each time to that at zero time are shown. (●) WT; (○) −1Ala; (■) −1Ser; (□) −1Val.

measured by the pulse chase method to the amount of mature HLY was also confirmed by measuring the amounts of mature HLY (M) and preHLY (P) in the exponentially grown cells from Western blots (not shown). The processing efficiency (the ratio of M/M+P) is summarized in table 1. The efficiency is remarkably low (6–41%) in the mutant signals which contain a statistically prohibited or a rare locating aa at position −1 or −3, and it is relatively high (75%) in the −2Pro mutant.

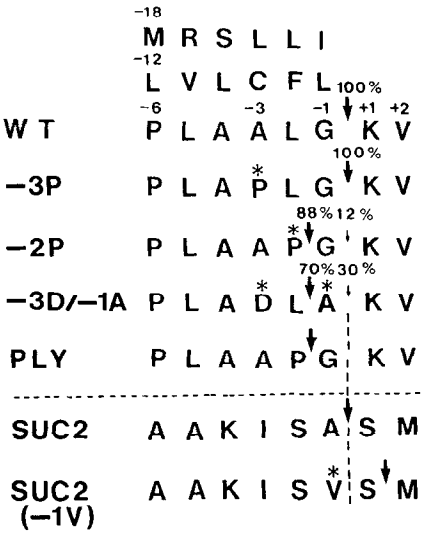

Fig.2. The signal cleavage sites in various preproteins. Amino acids are shown as capital letters and the positions of replaced amino acids are shown by asterisks. The cleavage sites and the ratios of each cleavage are shown by arrows and percentages, respectively. WT, wild type preHLY containing a native CLY signal; −3P, −3Pro mutant; −2P, −2Pro mutant; −3D/−1A, −3Asp/−1Ala mutant; PLY, pheasant prelysozyme; SUC2, yeast preinvertase; SUC2 (−1V), mutant preinvertase containing Val at position −1.

3.3. *Cleavage site determination*

HLYs processed from the preHLYs containing such statistically prohibited signals as −3Pro, −2Pro, and −3Asp/−1Ala, were purified to confirm their cleavage sites. The unique site identical to that of the native signal was found in the −3Pro signal, suggesting the acceptance of Pro at position −3 in yeast. Heterologous cleavages were found in −2Pro and −3Asp/−1Ala signals (fig.2). The major cleavage site of the −2Pro signal (88%) and the −3Asp/−2Ala signal (70%) was shifted one aa closer to the N-terminal side than that of the native signal, although the minor site (12–30%) was identical to that of the native signal.

4. DISCUSSION

The same shifts in the main cleavage site were found in −2Pro and −3Asp/−1Ala signals, despite the great differences in amounts of HLY secreted (265 μg/l for −2Pro and 1.7 μg/l for −3Asp/−1Ala mutants). The −3Pro signal commanded a poor secretion of HLY (8.8 μg/l), but was processed at the same site as the native signal. The computer program, SIGSEQ2 [11], based on a statistic weight-matrix method [12], was applied for the mutant signals to evaluate the most probable site of cleavage. The actual major site of cleavage for −2Pro and −3Asp/−1Ala signals has the highest processing probability, but that for the −3Pro signal had the third highest score (not shown), suggesting that this method is insufficient to predict the processing site of the statistically rate aa sequences.

The cleavage site of the −2Pro mutant was identical to that of pheasant prelysozyme, which contains the same signal sequence as the −2Pro signal [13]. This suggests that the signal peptidase is similar in yeast and bird and prefers the −3Ala/−1Pro sequence to the native −3Ala/−1Gly sequence. The −3Asp/−1Ala mutant containing a negatively charged aa at position −3 resembles (with respect to the poor processing and the shift in the cleavage site [14], although the sites of cleavage are different (fig.2)) the yeast mutant preinvertase containing a bulky aa (Val) at position −1 instead of the native Ala. It is probable that the signal peptidase is able to choose a suitable cleavage site when the original sequence is not fit to cleave.

Acknowledgement: We are grateful to Dr Rodney J. Folz for providing the computer program, SIGSEQ2, to evaluate the cleavage site.

REFERENCES

[1] Jigami, Y., Muraki, M., Harada, N. and Tanaka, H. (1986) Gene 43, 273–279.
[2] Von Heijne, G. (1983) Eur. J. Biochem. 133, 17–21.
[3] Von Heijne, G. (1986) Nucleic Acids Res. 12, 505–519.
[4] Nogi, Y., Shimada, H., Matsuzaki, Y., Hashimoto, H. and Fukasawa, T. (1984) Mol. Gen. Genet. 195, 29–34.
[5] Sherman, F., Fink, G.R. and Hicks, J.D. (1986) in: Methods in Yeast Genetics, p.164, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[6] Muraki, M., Jigami, Y., Morikawa, M. and Tanaka, H. (1987) Biochim. Biophys. Acta 911, 376–380.
[7] Taylor, J.W., Ott, J. and Eckstein, F. (1985) Nucleic Acids Res. 13, 8764–8785.
[8] Uemura, H., Shiba, T., Paterson, M., Jigami, Y. and Tanaka, H. (1986) Gene 45, 67–75.
[9] Summer-Smith, M., Bozzato, R.P., Skipper, N., Davies, R.W. and Hopper, J.E. (1985) Gene 36, 333–340.
[10] Laemmli, U.K. (1970) Nature 227, 680–685.
[11] Folz, R.J. and Gordon, J.I. (1987) Biochem. Biophys. Res. Commun. 147, 870–877.
[12] Von Heijne, G. (1986) Nucleic Acids Res. 14, 4683–4690.
[13] Weisman, L.S., Krummel, B.M. and Wilson, A.C. (1986) J. Biol. Chem. 261, 2309–2313.
[14] Schauer, I., Emr, S., Gross, C. and Schekman, R. (1985) J. Cell Biol. 100, 1664–1675.