A novel yeast secretion signal isolated from 28K killer precursor protein encoded on the linear DNA plasmid pGKL1

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ABSTRACT

Saccharomyces cerevisiae harboring linear dsDNA plasmids, pGKL1 and pGKL2, secretes a killer toxin consisting of 97, 31 and 28 kilodalton subunits (Nucleic Acids Res., 15, 1031-1046, 1987). We isolated the DNA encoding the N-terminal pre-sequence of the 28K precursor protein and constructed a new secretion vector in *S. cerevisiae*. Mouse α -amylase fused to the 28K signal sequence was secreted into the culture medium with a high efficiency similar to those fused to the mating factor α and 97K-31K killer signal sequences. This data clearly indicates that 28K pre-sequence functions as a secretion signal. Glycosylated and non-glycosylated α -amylase molecules were detected in the culture medium. The secretion of α -amylase was blocked by *sec18-1* mutation. The secreted α -amylase recovered from the medium was found to migrate faster in SDS-polyacrylamide gel than the precursor form of α -amylase synthesized *in vitro*. These lines of evidence suggest that mouse α -amylase fused to 28K killer signal sequence was processed, glycosylated and secreted through the normal secretion pathway of the yeast.

INTRODUCTION

The yeast *Saccharomyces cerevisiae* has a number of advantages as a host for the production of foreign proteins by the recombinant-DNA techniques (1, 2). In particular, since *S. cerevisiae* secretes a restricted number of proteins into the medium, foreign proteins expressed in the yeast cytoplasm could be secreted to the culture medium through the yeast secretion pathway by utilizing appropriate secretion signals. Many heterologous signal sequences from mammalian (3), plant (4) and fungal (5) secreted proteins and the homologous one isolated from yeast proteins, e. g. mating factor α , invertase and acid phosphatase have been used for the construction of yeast secretion vectors (1, 6). In terms of the efficiency of secretion and the accuracy of processing, the yeast homologous

secretion signals are superior to heterologous signals (1, 7-10). However, the number of secretion signal isolated from yeast proteins is rather limited, and the identification of new yeast secretion signals is important for the development of product secretion system in yeast.

We and other workers have reported that *S. cerevisiae* and other yeast cells harboring the linear DNA plasmids, pGKL1 and pGKL2, secrete a killer toxin consists of 97K, 31K and 28K subunits to the culture medium (11-14). According to the DNA sequence analysis of pGKL plasmid (11-13), the precursor form of two larger subunits, 97K and 31K, were encoded on ORF2 and that of 28K subunit was on ORF4, respectively (Fig. 1A). The precise secretion mechanisms of this killer toxin have not been studied well.

One interesting observation that the secretion of not only 97K and 31K subunits but also 28K subunit was stopped by the deletion of ORF2 region of pGKL1 has been reported by Stark et al(13). This data implies that the secretion of 28K subunit might require the co-secretion of other subunits, and the N-terminal pre-sequence of 28K subunit might not function as a secretion signal solely. The determination of N-terminal 28K pre-sequence as a secretion signal (signal peptide) remains to be examined.

We previously reported the construction of yeast secretion vector utilizing the secretion signal isolated from 97K and 31K killer precursor protein (16).

In the present paper, we described the isolation of a pre-sequence from 28K killer precursor protein, and the construction of new efficient yeast secretion vector. The characterization of this secretion vector clearly indicates that the pre-sequence of 28K subunit functions as a secretion signal.

MATERIALS AND METHODS

Yeast strains and medium

S. cerevisiae 20B12KR16 (α , trp1, pep4-3, killer^R) and XMF6-1 (α , sec18-1, trp1, pep4-3) were described previously (16). SD minimal medium (0.67% yeast nitrogen base and 2% glucose) and YCD rich medium (1% yeast extract, 2% casamino

acids and 2% glucose) were used. YPS plate (1% yeast extract, 2% Bacto-peptone, 1% soluble starch and 2% agar) was used for the halo assay of the secreted α -amylase.

Construction of pNW052

The DNA manipulation was carried out by the methods of Maniatis et al (17). The RsaI-PstI fragment (113 bp), containing 28K signal sequence, was cut out from pNW002 (14) and ligated with HincII-PstI digested pUC12 to construct pNW043. Plasmid pNW043 was digested with BamHI and subsequently with BAL31 up to 33 bp upstream of initiation codon of pro28K (ORF4) protein. This digested plasmid was religated with BglII linker. Plasmid pNW048, thus obtained, was digested with BglII-PstI in order to isolate the 86 bp of DNA fragment encoding signal sequence of pro28K protein. This fragment and the EcoRI-BglII fragment (1.5 kb) isolated from pRYG1 encoding PGK (phosphoglycerate kinase) promoter region, were subcloned into EcoRI-PstI digested pUC12 to construct pNW051. The EcoRI-PstI fragment (1.6 kb) of pNW051 and the PstI-BamHI fragment (1.5 kb) of pNW050 encoding mouse α -amylase without its own signal sequence were ligated into EcoRI-BglII digested E. coli-yeast shuttle vector pMT57 to construct pNW052 (Fig. 2).

Other plasmids

Plasmids pREI082, pMT56, and pREI052 were described previously (16). Plasmid pNW047 containing a 97K-31K secretion signal was constructed by the insertion of transcription termination signal of PGK gene after amylase coding region of pNW037 (16).

In vitro transcription and translation

The DNA fragment (1.6 kb) encoding mouse α -amylase fused to the 28K signal sequence was cut out from pNW052 by BglII and SspI, and inserted into the BamHI-SmaI site of pGEM1 (Promega Biotec) to construct pNW059.

The mRNA for the 28K signal sequence- α -amylase fusion gene was transcribed in vitro for 1 hr at 40°C in the following mixture (20 μ l); 80 mM Tris-HCl buffer (pH 7.5), 24 mM MgCl₂, 8 mM spermidine, 10 mM DTT, 40 units of RNasin (Promega Biotec), 0.5 mM each of ATP, CTP and UTP, 0.1 mM GTP, 1 mM m⁷G(5')ppp(5')G, 2 μ g of SstI- digested pNW059 DNA, and 15 units

of SP6 polymerase. Reaction was stopped by the phenol-CHCl₃ extraction and mRNA was precipitated by ethanol and dissolved in 40 µl of H₂O.

In vitro translation was carried out for 30 min at 25°C in the following reaction mixture (30 µl); 67 mM potassium acetate, (173 mM of final potassium concentration in mixture), 0.067 mM amino acids mixture minus Met, 15 µCi [³⁵S]-Met, 15 µl of wheat germ extract (Amersham) and 1 µl of in vitro mRNA described above.

The reaction was stopped by the addition of Laemmli sample buffer (18) and heated for 3 min at 95°C. The translation products were analyzed by SDS-gel electrophoresis with autoradiography.

Others

DNA sequence was determined by the dideoxy chain termination method (TOYOBO M13 sequencing kit). Yeast transformation was carried out by the method of Ito et al (19). Anti-serum and immuno-blotting were described previously (16).

RESULTS AND DISCUSSION

Construction of secretion vector using the pre-sequence of 28K killer precursor protein

Two different types of pre-sequences were found at the N-terminal region of each of the precursors of the pGKL killer subunits. Prepro-97K-31K protein (ORF2) has a secretion signal of 29 amino acids long consisting of a 17 hydrophobic amino acids stretch followed by relatively hydrophilic 12 amino acids (12). This prepro-signal was finally cleaved at the carboxyl side of Lys-Arg residues most likely by the KEX2 gene product.

The 28K killer subunit (ORF4) was first synthesized as a larger precursor with 18 amino acids N-terminal extension (Fig. 1B. underlined). This 18 amino acids extension shares several major structural homologies with signal sequence of prokaryotic precursor proteins. There are two basic amino acids, Lys and His, at the N-terminal region, followed by a stretch of relatively hydrophobic amino acids including Ser and Thr, and the cleavage site of processing enzyme is at Ala-X. The 28K precursor was found to be cleaved by putative yeast signal

Fig. 1.A. Open reading frames (ORFs) identified on pGKL1 plasmid.

The killer subunits 97K and 31K are encoded on ORF2 and 28K is encoded on ORF4. Immunity protein is encoded on ORF3. ORF1 and ORF2 in previous report (12) were corrected to be a single ORF1 here (Hishinuma, F., unpublished results, 15). We used here the same nomenclature of ORFs as Stark *et al.* (13). The open boxes in the killer structure represent the signal sequences of the precursor proteins.

Fig. 1.B. DNA and deduced amino acid sequences in the 5'-flanking and 5'-coding regions of pGKL killer 28K signal sequence-mouse α -amylase fused gene.

The DNA underlined is derived from linear plasmid pGKL1 and encodes 28K signal sequence. Broken line represents the N-terminal truncated mouse α -amylase coding region. The 23rd Pro residue corresponds to the 19th residue of the native mouse α -amylase precursor. The killer 28K precursor protein was reported to be cleaved between 18th and 19th Ala residues (13). The first nucleotide A in the DNA sequence corresponds to -3 position of mRNA start site of PGK gene.

peptidase between 18th and 19th Ala residues (Fig. 1B.)(13). The PstI restriction enzyme site on the DNA sequence was found at this precursor processing site (12). This is a convenient property useful for the construction of secretion vector for the expression of foreign proteins.

We isolated DNA fragment encoding the first 19 amino acid residues of the 28K killer toxin, and constructed the secretion vector, pNW052, in order to measure the secretion efficiency of this pre-sequence (see Materials and Methods, Fig. 2). The DNA

Fig. 2. Construction of α -amylase secretion vector pNW052. , 28K signal sequence; , PGK promoter region; , α -amylase.

fragment encoding the 28K pre-sequence was fused to the mouse α -amylase gene without its own signal sequence. The truncated α -amylase without the signal sequence was shown not to be secreted from the yeast cells without the aid of a signal sequence (6). The **PGK** promoter (20), one of the strongest promoters in *S. cerevisiae*, was used for the expression of this fused gene. The DNA and deduced amino acid sequences of the 5'-flanking and 5'-coding regions of the fused protein were shown in Fig. 1B.

To measure the α -amylase activity secreted from the yeast cells, *S. cerevisiae* strain 20B12KR16 was transformed with

Fig. 3. Halo-assay of secreted α -amylase activity.

S. cerevisiae 20B12KR16 transformants were patched on YPS agar plate and incubated for 48 hr at 30°C. Undigested starch was stained with I_2 -KI solution.

secretion vectors, and transformants were patched on a YPS agar plate. When α -amylase was secreted from the cells, it digested starch, forming a halo around the amylase-positive cells. The halo was clearly observed by the staining of undigested starch with I_2 -KI solution. As shown in Fig. 3, 20B12KR16(pNW052) formed a large clear halo of a size comparable to that of strain carrying pMT56, indicating that 28K pre-sequence functions as a secretion signal and is as efficient as the most frequently used MF α 1 leader sequence in directing mouse α -amylase to be secreted into the medium. The halo size of 20B12KR16(pNW052) is much larger than that of 20B12KR16(pREI082) which encodes mouse α -amylase with its own signal sequence as a secretion signal. These data clearly indicate that the homologous yeast secretion signal is recognized by the yeast secretion machinery much more efficiently than the heterologous one. The halo formed by 20B12KR16(pNW047) was also shown in Fig. 3. Plasmids pNW037 (16) and pNW047 are the α -amylase secretion vectors using pGKL killer 97K-31K signal sequence for the secretion signal. The insertion of the PGK 3'-flanking region encoding PGK transcription termination signal downstream of the α -amylase gene in pNW037 to obtain pNW047 improved the secretion efficiency to 120% of the

Fig. 4. Identification of α -amylase secreted in the culture medium by SDS-polyacrylamide gel electrophoresis and immuno-blotting.

TCA precipitated total proteins in 1 ml of culture medium were applied onto 12% SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue (lanes 1 through 5) and with anti-human α -amylase antiserum (lanes 6 through 9). Lanes 1 and 6, molecular weight standards of phosphorylase b (97 kd), bovine serum albumin (66 kd), and ovalbumin (45 kd), each 1 μ g; lanes 2 and 7, culture medium of 20B12KR16(pNW052), lanes 3 and 8, culture medium of 20B12KR16(pREI052), lanes 4 and 9, culture medium of 20B12KR16(pNW037); lane 5, authentic human salivary α -amylase (Sigma IX-A).

original value. No halo was observed by 20B12KR16(pREI052) as a control. The amount of α -amylase secreted from 20B12KR16(pNW052) into the YCD liquid medium in jar fermenter is 1.2 mg/liter/18 hrs. Under these conditions, 94% of total α -amylase was secreted into the medium (average value of 4 times experiments).

Identification of mouse α -amylase in the culture medium of *S. cerevisiae* transformants

For the identification of mouse α -amylase secreted into the culture medium, the total proteins in 1ml of the culture medium was precipitated with 10% TCA, and analyzed by SDS-gel electrophoresis and immuno-blotting (Fig. 4). An extra band with a molecular weight of about 55,000 was observed in the culture medium of 20B12KR16(pNW052)(lane 2, shown by arrow

Fig. 5. Comparison of α -amylase made in the presence or absence of tunicamycin.

S. cerevisiae 20B12KR16(pNW052) was grown in the presence (lane 1) and absence (lane 2) of tunicamycin (1 μ g/ml culture) at 24°C. Total TCA precipitates in culture medium were analyzed by SDS-gel electrophoresis and immuno-blotting.

head), but not in the control 20B12KR16(pREI052)(lane 3). To verify that this extra band corresponds to α -amylase, we carried out the immuno-blotting with rabbit anti-human α -amylase anti-serum (16). The anti-human α -amylase anti-serum was cross-reactive to mouse α -amylase since these two α -amylases have a 80% homology in amino acid sequence (21, 22). As shown in Fig. 4, lane 7, we identified the mouse α -amylase molecules in the culture medium of 20B12KR16(pNW052); two bands with the mobility of the faster one exactly the same as the extra band in lane 2, were stained by anti- α -amylase anti-serum. The α -amylase secreted from 20B12KR16(pNW037), using 97K-31K killer signal for the secretion (16), were also shown in lane 4 and 9. The authentic human salivary α -amylase (Sigma IX-A) which was used for the preparation of anti-serum was shown in lane 5.

Since two potential glycosylation sites were observed in the amino acid sequence of mouse α -amylase (21), we assumed that the two α -amylase species secreted in the 20B12KR16(pNW052) culture medium were due to the heterogeneity of glycosylation during the secretion. 20B12KR16(pNW052) cells were grown in the presence of tunicamycin (1 μ g/ml culture) at 24°C overnight, and the α -amylase secreted to the medium was analyzed by immuno-

Fig. 6. Effects of sec18-1 mutation on the secretion of α -amylase.

S. cerevisiae 20B12KR16(pNW052)(SEC18-1) and XMF6-1(pNW052)(sec18-1) were patched on YPS agar plate and incubated at either 23°C or 37°C for 48 hr. Undigested starch was stained with I₂-KI solution to observe halo.

blotting. As shown in Fig. 5, lane 1, the slower migrating band disappeared, with only the faster migrating one observed in the presence of tunicamycin. Tunicamycin is the well-known antibiotic which inhibits N-glycosylation of secretory proteins due to its inhibition of the formation of dolichyl-pyrophosphoryl-N-acetylglucosamine (23). At a lower growth temperature (24°C), only nonglycosylated protein was reported to be secreted to the culture medium in the presence of tunicamycin (24). We conclude that the faster moving band corresponds to non-glycosylated α -amylase and the slower one to glycosylated enzyme.

Effect of sec mutation on the secretion of α -amylase

Shekman and his coworkers isolated a number of temperature-sensitive secretion-defective mutants (25). Protein secretion is pleiotropically inhibited in such mutants grown at the nonpermissive temperature (37°C), but is normal at the permissive temperature (23°C). The protein secretion in sec18 mutant was blocked at a step between the endoplasmic reticulum and the Golgi apparatus at 37°C.

Plasmid pNW052 was used to transform sec18-1 mutant (strain XMF6-1) and the effect of the sec18-1 mutation on the secretion of α -amylase was studied. As shown in Fig. 6, a large halo was observed around the XMF6-1(pNW052) cells at 23°C but not at

Fig. 7. Mobilities of the precursor form and the secreted α -amylase molecules on SDS-polyacrylamide gel electrophoresis.

Lane 1, autoradiogram of [35 S]-Met-labeled in vitro translation products directed by pNW059 mRNA; lane 2, Coomassie blue staining of total TCA precipitates in culture medium of 20B12KR16(pNW052).

37°C, indicating that the α -amylase secretion was blocked by the sec18-1 mutation. As a control, SEC wild type strain 20B12KR16(pNW052) formed large halos at both 23°C and 37°C. These data suggest that the secretion of mouse α -amylase encoded on plasmid pNW052 follows the normal yeast secretion pathway.

Processing of α -amylase precursor

Since the secretion of α -amylase from 20B12KR16(pNW052) appears to follow the normal secretion pathway in the yeast (Fig. 6), it might be expected that the signal sequence of the precursor could be removed during the translocation across the ER membrane. The precursor form of α -amylase (28K signal sequence- α -amylase fused protein) was prepared in an in vitro transcription-translation system. The DNA fragment encoding the fused-gene was inserted downstream of SP6 promoter (pNW059, see Materials and Methods), and the resulting clone was used as the

template for transcription in vitro. The mRNA, thus obtained, was translated in the wheat germ extract in the presence of [³⁵S]-Met. The [³⁵S]-Met labeled precursor form of α -amylase synthesized in vitro (Fig. 7, lane 1) and the secreted α -amylase recovered in the medium of 20B12KR16(pNW052)(lane 2) were analyzed by SDS-PAGE with autoradiography and Commasie blue staining, respectively. As shown in Fig. 7, lane 1, one major band (shown by arrow) was observed at a molecular weight around 55,000. This band was immunoprecipitable with anti- α -amylase anti-serum (data not shown). The mobility of the precursor form of α -amylase in lane 1 was slightly slower than that of α -amylase molecule secreted in the medium (lane 2, shown by arrow), suggesting that the signal sequence of the precursor was removed during the secretion process. However, the N-terminal amino acid analysis of the secreted α -amylase was not carried out.

CONCLUSION

We have constructed a novel yeast secretion vector using the signal sequence in the precursor form of pGKL killer 28K subunit. The 18 amino acids signal sequence in 28K killer toxin contains sufficient information to direct the secretion of mouse α -amylase to the culture medium of S. cerevisiae.

The efficiency of this new signal for the secretion of α -amylase is compatible to that of MF α 1 leader sequence and pGKL killer 97K-31K pro-sequence which we reported previously (16). The killer toxin system encoded on pGKL plasmids was proved to be a good source of secretion signal for the construction of secretion vectors in yeast.

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