Degradation of Rhizopus niveus Aspartic Proteinase-I with Mutated Prosequences Occurs in the Endoplasmic Reticulum of Saccharomyces cerevisiae*

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Rhizopus niveus aspartic proteinase-I (RNAP-I) is secreted by Saccharomyces cerevisiae extracellularly (Horiuchi, H., Ashikari, T., Amachi, T., Yoshizumi, H., Takagi, M., and Yano, K. (1990) Agric. Biol. Chem. 54, 1771–1779). The prosequence of RNAP-I has the function to promote correct folding of its mature part. Deletion (Δpro) and amino acid substitutions (M1) in the prosequence of RNAP-I (Fukuda, R., Horiuchi, H., Ohta, A., and Takagi, M. (1994) J. Biol. Chem. 269, 9556–9561).

In this study, little accumulation of Δpro was observed in Western blot analysis of the cell extracts of the transformants producing Δpro using anti-RNAP-I antisera. In contrast, M1 was accumulated in the yeast cells. Pulse-chase analysis revealed that they were synthesized at almost the same rates and that Δpro was degraded in the cells more rapidly than M1. In subcellular fractionation analysis, Δpro was found in the fraction that contained most of the activity of an endoplasmic reticulum (ER) marker enzyme, NADPH-cytochrome c reductase. In indirect immunofluorescence microscopy, Δpro was observed in the ER. Similar result was also observed in a mutant which is deficient of the two vacuolar proteases, proteinase A and proteinase B. So, the vacuolar proteases are not involved in degradation of Δpro. From these results, we concluded that RNAP-Is with the mutated prosequences, which probably could not be folded correctly, were retained and degraded in the ER.

In eukaryotic cells, most of nascent secretory proteins are targeted to the endoplasmic reticulum (ER)† after their signal sequences are recognized. Then these proteins are translocated across the ER membrane co- or post-translationally. Many secretory proteins have their final conformations in the ER lumen, then are transported to the Golgi compartment and secreted from cells. Some proteins that cannot be folded correctly or assembled into proper multimers are retained in the ER. Molecular chaperones such as immunoglobulin heavy chain binding protein, BiP, bind to those immature or abnormal proteins and keep them retained in the ER to stimulate their proper folding or multimerization (for review, see Refs. 3–6).

Recently, it has been revealed that protein degradation machinery is present in the ER and some secretory or membrane proteins that could not be folded or multimerized correctly are degraded in the ER (for review, see Ref. 7). But little is known about the mechanisms of recognition and degradation of malformed proteins by these protein degradation machinery in the ER. Three kinds of mutated proteins are shown to be degraded in the ER of Saccharomyces cerevisiae: these are a secretion-defective mutant of human α-1-protease inhibitor (8), mutants of carboxypeptidase Y, carrying an Arg instead of a Gly in a highly conserved region, and proteinase A, in which 37 amino acids spanning the processing site of the prosequence are deleted (9). In addition, it is considered that the unassembled 100-kDa integral membrane subunit of the yeast vacuolar H⁺-ATPase complex is degraded in the ER of the cell lacking Vma21p, which is suggested to be required for assembly of the integral membrane sector of V-ATPase in the ER (10).

Rhizopus niveus, a filamentous fungus, secretes large amounts of aspartic proteinases extracellularly. We have cloned and sequenced genes encoding aspartic proteinase-I (11) and aspartic proteinases II–V (RNAP-I to RNAP-V) (12). RNAP-I is synthesized as a precursor form with a pre-sequence (21 amino acid residues) and a prosequence (45 amino acid residues) at the N terminus of the mature part (323 amino acid residues). RNAP-I is also secreted extracellularly by S. cerevisiae efficiently when the encoding gene is introduced and expressed (1). The prosequence of RNAP-I is essential for both renaturation of the denatured mature part in vitro and secretion of the mature part in vivo in S. cerevisiae. Some RNAP-Is with the mutated prosequences are not secreted extracellularly at all (2). Therefore, the prosequence of RNAP-I is suggested to be essential for correct folding of its mature part in vivo.

In this paper, we examined the transport, accumulation, and degradation of those RNAP-Is with the mutated prosequences in S. cerevisiae. We found that these precursor RNAP-Is were retained and degraded in the ER. Therefore, it is concluded that the precursors that cannot be folded correctly in the ER lumen are specifically recognized and degraded by the degradation machinery in the ER lumen.

MATERIALS AND METHODS

Strains and Media—E. coli JA221 (recA1 leuB6 trpE6 hsdR7 supE44 recA1 leuB6 trpE6 hsdM’damaC1 13) was used as a host for plasmid construction and strain CJ236 (dtu1 ung1 thy1 recA1 leu2C105 F’cam+) as a host for site-directed mutagenesis.

S. cerevisiae strain R27–7C-1C (MATa his3 leu2 ura3 trp1) was used as a host for production of RNAP-I with the wild-type or the mutated prosequences. Strain #334 (MATa leu2 ura3 reg1 gal1 pep4 prb1) were used as hosts for production of Δpro. Yeast cells were cultivated aero-

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The abbreviations used are: ER, endoplasmic reticulum; HSP, high speed pellet; HSS, high speed supernatant; LSP, low speed pellet; PAGE, polyacrylamide gel electrophoresis; PDI, protein disulfide isomerase; RNAP-I, R. niveus aspartic proteinase-I; kb, kilobase(s).
bically at 30 °C. For selection of yeast transformants, YNBD medium (0.67% yeast nitrogen base without amino acids, 2% glucose) was used with appropriate supplements.

Enzymes and Reagents—Nucleic acid modification enzymes were purchased from Takara Shuzo Co. (Kyoto, J. apam) and used under conditions recommended by the manufacturer. An oligonucleotide for site-directed mutagenesis was synthesized with a model 391 DNA synthesizer (Applied Biosystems).

Plasmid Construction—Recombinant DNA manipulations were done by the standard methods (13). Yeast transformation was carried out using the lithium acetate procedure described by Ito et al. (14).

Plasmid pYPR28412, which encodes an RNA-P-derivative Apro* in which Asp100 of Apro was replaced with Ala by site-directed mutagenesis, was constructed as follows. A 0.4-kb EcorI-Sall fragment from pYPR2841 (2) was cloned between EcoRI and Sall sites of pUC19 (resulting in pUC2841), and Apro* was replaced with Ala by site-directed mutagenesis with a 26-mer synthetic oligonucleotide, 5'-AACCTGATAATTCACGATATCC-3'. A 0.4-kb EcoRI-Sall fragment from the resultant double-stranded DNA was ligated with a 9.4-kb EcoRI-Sall fragment of pYPR2841 to form the plasmid pYPR28412.

Plasmid pYPR2841U, which was used to express Apro in pep4 prb1 cells, was constructed as follows. A 2.2-kb HindIII fragment of pYPR2841, which contained glyceraldehyde-3-phosphate dehydrogenase gene promoter, Apro, and glyceraldehyde-3-phosphate dehydrogenase gene terminator for Apro, was transferred into the site of YEp24.

Accurate construction of all plasmids was confirmed by nucleotide sequencing.

Nucleotide sequencing was performed on the Applied Biosystems model 373A DNA sequencing system.

Preparation of Total Cell Extracts—Yeast cells were collected in a tube, washed with 10 mM sodium azide, and suspended in Laemmli sample buffer (15). One volume of glass beads was added to the tube, and then it was vortexed for 20 s and put on ice for 1 min. This step was repeated five times, and the preparation was boiled for 5 min. After centrifugation at 15,000 rpm for 5 min at 4 °C, the supernatant was used as a total cell extract.

Western Blot Analysis—SDS-PAGE was done by the method of Amersham Corp. (15). Detection of filter-bound antibodies was carried out according to the enhanced chemiluminescence method with ECL detection reagents (Amersham Corp.).

Pulse-chase Analysis—Transformants of S. cerevisiae were cultured in YNBDCU medium (YNB medium containing 2% camagino acids and 50 μg/ml uracil) for 12 h. Cells in a 1.1-ml culture were collected and resuspended in 1.1 ml of YNBDCU medium (YNB medium containing 20 μg/ml histidine, 30 μg/ml leucine, and 50 μg/ml uracil) supplemented with the amino acid mixture (20 μg/ml arginine, 30 μg/ml tyrosine, 30 μg/ml isoleucine, 30 μg/ml lysine, 50 μg/ml phenylalanine, 100 μg/ml glutamic acid, 100 μg/ml aspartic acid, 150 μg/ml valine, 200 μg/ml threonine, 400 μg/ml serine). After 20 min at 30 °C, cells were harvested, resuspended with 0.1 M Ci of [35S]methionine for 5 min and an aliquot (200 μl) was transferred to a chilled tube containing 2 μl of 100 mM azide. Then the remaining cells were collected, washed, and incubated in 900 μl of YNBDCU medium supplemented with the amino acid mixture and 2 mg/ml methionine. An aliquot was removed into ice-cold azide at intervals.

Immunoprecipitation was performed by the method described by Franzensoff et al. (16) with some modifications. Total cell extract (40 μl) was prepared as described by Yaffe and Schatz (17). Four volumes (160 μl) of IP dilution buffer (1.25% Triton X-100, 190 mM NaCl, 6 mM EDTA, 60 mM Tris-HCl (pH 7.4)) were added to the cell extract in a tube. After the addition of 4 μl of 1:10 diluted preimmune sera, the extract was incubated on ice for 1 h. Then 40 μl of 10% IgG Sorb (The Enzyme Center, Inc., Malden, MA) were added, and the extract was incubated for 30 min on ice. After centrifugation at 10,000 × g for 5 min, the supernatant was taken into a tube and 1:10 diluted anti-RNAP-I antiserum was added, after which it was incubated for 1 h on ice. Then 40 μl of 10% IgG Sorb was added, and the extract was incubated for 30 min on ice. After centrifugation, the pellet in the tube was washed four times with IP buffer (1% Triton X-100, 0.2% SDS, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4)). After 40 μl of Laemmli sample buffer was added, it was boiled for 5 min and the mixture was subjected to SDS-PAGE.

Subcellular Fractionation Analysis—Subcellular fractionation analysis was performed by the method of Nakano et al. (18) with some modifications.

S. cerevisiae spheroplasts were prepared by the method of Franzensoff et al. (16). Spheroplasts were disrupted with Potter-Elvehjem homogenizer (15 strokes). The extracts were centrifuged at 1,000 × g for 10 min at 4 °C to prepare the pellet (low speed pellet, LSP) and the supernatant (low speed supernatant). The low speed supernatant fraction was further centrifuged at 100,000 × g for 1 h at 4 °C to obtain the high speed pellet (HSP) and the supernatant (HSS). The amount of Apro or M1 was examined by Western blot analysis with anti-RNAP-I antisera.

RESULTS

RNAP-Is with the Mutated Prosequences Were Degradated in the Cells—The presence of RNAP-I is essential for extracellular secretion of the mature part in S. cerevisiae and RNAP-Is with the mutated prosequences, Apro and M1, are not secreted at all (2). In Apro, the whole prosequence (from Ser22 to Ala66) is deleted, and in M1, Ala49-Leu50 is replaced by Asp-Pro (numbers are from the initiation codon Met).

To examine whether these RNAP-Is with the mutated prosequences were synthesized in the cells, the transformants harboring the plasmids encoding these RNAP-Is with the mutated prosequences were cultured for 14 h at 30 °C, and the total cell extracts were prepared and subjected to Western blot analysis using anti-RNAP-I antisera. Both of the mutated as well as the wild-type RNAP-I precursors were detected in the cell extracts, but not in equal amounts. Little accumulation of Apro was observed in the extracts of the cells (Fig. 1, lane 3), and the amount of M1 accumulated in the cells was more than that of Apro (Fig. 1, lanes 3 and 4). To test the possibility of autocatalytic degradation of Apro in the cells, the intracellular accumulation of Apro, in which one of the active sites, Asp100* of Apro, was deleted to Al to inactivate this enzyme was examined. The amount of Apro* in the cells was less than that of the wild-type precursors and was almost equal to that of...
Δpro (Fig. 1, lanes 2, 3, and 5). Therefore, it was suggested that lower level of accumulation of Δpro was not due to autocatalytic degradation in the cells.

To investigate why Δpro was not accumulated so much as M1 in the cells, the pulse-chase analysis was done. Transformants producing Δpro or M1 were pulse-labeled for 5 min with [35S]methionine and chased for 0, 30, or 60 min. Δpro and M1 were synthesized at almost the same levels (Fig. 2, lanes 1 and 4). However, after 30 min of chase, most of Δpro disappeared from the cell extract (Fig. 2, lane 2). The amount of M1 after 30 min of chase also decreased, but not so much as that of Δpro did (Fig. 2, lane 5). Δpro and M1 were not secreted extracellularly at all (2). These results indicated that Δpro and M1 were degraded in the cells and that the lower level of accumulation of Δpro in the cells was due to the faster rate of the intracellular degradation.

Δpro and M1 Were Degraded in the ER—To determine the intracellular site of accumulation of Δpro and M1, subcellular fractionation analysis was performed. Spheroplasts of the transformants producing Δpro or M1 were homogenized, and the cell extracts were subjected to differential centrifugation at 10,000 g and 100,000 g. Three fractions, LSP, HSP, and HSS, were analyzed by Western blotting with anti-RNAP-I antisera. The activity of an ER marker enzyme, NADPH-cytochrome c reductase, was also collected in the HSS fraction. Therefore both Δpro and M1 were recovered exclusively in the LSP fraction (Table I). Most of the activity of an ER marker enzyme, NADPH-cytochrome c reductase, was also collected in the LSP fraction. The activity of a Golgi marker enzyme, heat-stable dipeptidylaminopeptidase, was recovered in both the LSP and HSP fractions. The activity of a vacuolar enzyme, carboxypeptidase Y, was recovered in both the LSP and HSS fractions. Most of the activity of cytosolic enzyme, α-galactosidase, was collected in the HSS fraction. Therefore both Δpro and M1 were obtained in the fractions that included most of the ER marker enzyme activity.

To further define the localization of Δpro, indirect immunofluorescence microscopy with anti-RNAP-I antisera was carried out. The nuclei in these cells were simultaneously stained with 4,6-diamidino-2-phenylindole. The transformants producing Δpro showed prominent staining of the perinuclear region when non-related antibodies (anti-β-galactosidase antibodies) were applied to those cells (Fig. 3C). The transformants producing M1 showed the same staining pattern as those producing Δpro (data not shown). Δpro was not accumulated so much as M1 in the cells, the pulse-chase analysis was done. Transformants producing Δpro or M1 were pulse-labeled for 5 min with [35S]methionine and chased for 0, 30, or 60 min. Δpro and M1 were synthesized at almost the same levels (Fig. 2, lanes 1 and 4). However, after 30 min of chase, most of Δpro disappeared from the cell extract (Fig. 2, lane 2). The amount of M1 after 30 min of chase also decreased, but not so much as that of Δpro did (Fig. 2, lane 5).

In eukaryotic cells, vacuole (lysosome in mammalian cells) contains various proteases and many proteins are degraded in it (23, 24). If Δpro is degraded in vacuole, Δpro would be accumulated in vacuole of the vacuolar protease-deficient cells. To address this possibility, the localization of Δpro was investigated in a mutant that is deficient of the major vacuolar proteases, proteinase A and proteinase B, by indirect immunofluorescence microscopical observation. In pep4 prb1 cells, prominent staining of the perinuclear region was observed as in the wild-type cells (Fig. 4A). The staining pattern of PDI was similar to that of Δpro (Fig. 4B). The staining pattern of Δpro was different from the pattern of vacuole visualized with lucifer yellow CH (Fig. 4D). Therefore, it was suggested that Δpro was degraded not in vacuole, but in the ER.

![Fig. 2. Degradation of Δpro and M1 in the cells.](image)

![Fig. 3. Δpro was retained in the ER.](image)

**Table I**

| Protein (localization) | % Total | ΔPro (LSP) | ΔPro (HSP) | ΔPro (HSS) | M1 (LSP) | M1 (HSP) | M1 (HSS) |
|------------------------|---------|------------|------------|------------|----------|----------|----------|
| ΔPro or M1             | 100     | 93         | 7          | 0          | 90       | 10       | 0        |
| NADPH-cytochrome c reductase (ER) | 70 | 19 | 11 | 60 | 22 | 18 |
| Heat-stable DPAP (Golgi) | 51 | 41 | 8 | 54 | 38 | 8 |
| Carboxypeptidase Y (vacuole) | 28 | 5 | 67 | 36 | 16 | 48 |
| α-Glucosidase (cytosol) | 16 | 4 | 80 | 24 | 13 | 63 |
Protein Degradation in the Endoplasmic Reticulum

Δpro is degraded more rapidly than M1. Δpro is deficient of the whole prosequence, while M1 has the only prosequence with 2 amino acids substituted. So, it is speculated that Δpro cannot have correctly folded structure in the ER lumen, whereas M1 may have partially folded structure. Therefore, Δpro may be more easily recognized or degraded by the degradation machinery in the ER than M1.

Based on the results described in this paper, we propose a model for the transport, accumulation, and degradation of RNAP-I precursors in the ER lumen of S. cerevisiae. The wild-type precursor has the correct folding structure by the function of the prosequence and is secreted extracellularly. In contrast, Δpro and M1 cannot be folded correctly because of the mutations in their prosequences and are degraded in the ER by an unknown degradation mechanism.

We are interested in the mechanism via which unfolded or malfolded proteins such as Δpro or M1 are recognized by the degradation machinery and in proteases that participate in the degradation of those proteins. We have isolated several mutants of S. cerevisiae that have deficiencies in the degradation of Δpro or accumulate Δpro in the cells, probably in the ER. It is expected that mutants defective in the recognition and/or degradation machinery in the ER would be present among them. Characterization of these mutants will be helpful to elucidate the mechanisms of recognition and degradation of proteins in the ER.

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FIG. 4. Δpro was retained in the ER of pep4 prb1 cells. A–C, right panel, indirect immunofluorescence microscopical observation in pep4 prb1 cells was performed using anti-RNAP-I antisera (A), anti-PDI antibodies (B), or non-related antibodies (anti-β-galactosidase antibodies) (C), respectively. Left panel, the nuclear DNA in these cells was also stained with 4′,6-diamidino-2-phenylindole. D, right panel, vacuolar components of pep4 prb1 cells were visualized with lucifer yellow CH. Left panel, the same cells were observed by light microscopy.

DISCUSSION

In this paper, we analyzed the intracellular transport, accumulation, and degradation of RNAP-Is with the mutated prosequences, Δpro and M1, that were not secreted from S. cerevisiae. Δpro and M1 were synthesized at almost the same rate, but Δpro was degraded more rapidly than M1 in the cells. Therefore, the amount of M1 accumulated in the cells was more than that of Δpro. The subcellular fractionation analysis showed that both Δpro and M1 were recovered in the fractions that contained most of the activity of the ER marker enzyme, NADPH-cytochrome c reductase. In addition, indirect immunofluorescence microscopy with anti-RNAP-I antisera demonstrated that Δpro localized in the ER. The staining pattern of Δpro did not change even in pep4 prb1 cells. Thus, it was presumed that vacuolar proteases did not take part in the degradation of Δpro. Consequently, we concluded that these RNAP-Is with the mutated prosequences were retained and degraded in the ER.

It was revealed that denatured RNAP-I could renature in the presence of the wild-type prosequence in vitro, but not in the absence of the prosequence or in the presence of that of M1 (2). Therefore, Δpro and M1 seem to be unable to have the correct tertiary structure in the ER lumen of S. cerevisiae. In this case, they would be recognized and degraded specifically by the degradation machinery in the ER. Our results presented in this paper support this hypothesis. However, it is not clear why Δpro is degraded more rapidly than M1. Δpro is deficient of the whole prosequence, while M1 has the only prosequence with 2 amino acids substituted. So, it is speculated that Δpro cannot have correctly folded structure in the ER lumen, whereas M1 may have partially folded structure. Therefore, Δpro may be more easily recognized or degraded by the degradation machinery in the ER than M1.
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