Role of the Proregion in the Production and Secretion of the Yarrowia lipolytica Alkaline Extracellular Protease*

(Received for publication, June 4, 1990)

Emmanuelle Fabré, Jean-Marc Nicaud, Maria Carmen López, and Claude Gaillard

From the Institut National Agronomique, Centre de Biotechnologie Agro-Industrielle, Laboratoire de Génétique, Institut National de la Recherche Agronomique, Centre National de la Recherche Scientifique, 78850 Thiverval, Grignon, France

The yeast Yarrowia lipolytica secretes an alkaline extracellular protease (AEP). It is first synthesized as a precursor comprising a putative signal peptide, a stretch of 10 X-Ala or X-Pro sequences that are substrates for a dipeptidyl aminopeptidase, a large proregion that contains a glycosylation site and two Lys-Arg sites that can be cleaved by a KEX2-like endoprotease and finally the mature protease itself. A defect in the XPRG (KEX2-like) gene results in the secretion of an inactive proenzyme (Matoba, S., and Ogrydziak, D. M. (1989) J. Biol. Chem. 264, 6037-6043), showing that the proregion inhibits protease activity. To determine whether the proregion plays an additional role in protease secretion, we have generated deletions and point mutations in the corresponding region of the structural gene. In this paper we examine the effects of these mutations on AEP secretion and maturation and show that the proregion is essential for its secretion. All deletions affecting the proregion resulted in the intracellular accumulation of unprocessed precursors. Deletion of the glycosylation site in the proregion resulted in the production of an unglycosylated precursor that was secreted and matured correctly at 18 °C but accumulated in the cells at 28 °C. From these results, we propose that the AEP prosequence plays an additional essential role in guiding the proper folding of the protein into a conformation compatible with secretion.

Although the roles of signal sequences and other signals involved in routing proteins to different cellular organelles are now well established (Pugsley, 1989), the functions of proregions such as those present in α-factor (Julius et al., 1983), carboxypeptidase Y (Valls et al., 1987), antibiotics such as nisin (Keletta and Entian, 1989), and several proteases (α-lytic protease (Silén et al., 1989), subtilisin (Ikemura et al., 1987), alkaline and neutral protease from Bacillus amyloliquefaciens (Nakayama et al., 1987; Vasantha et al., 1984), alkaline extracellular protease (AEP) from the yeast Yarrowia lipolytica (Davidow et al., 1987), and from lactic bacteria (Kok et al., 1975) are less well understood. The length of the proregion may vary from a few amino acids (seven for α-lytic protease; Silén et al., 1989) to several hundred (e.g. 221 amino acids for the neutral protease from B. amyloliquefaciens (Vasantha et al., 1984) and 143 amino acids for the AEP (Davidow et al., 1987)). In addition, proregions may carry sites for post-transcriptional modifications such as glycosylation (e.g. one in the AEP propeptide and three in the α-factor propeptide). It has been proposed that propeptides may prevent the formation of active structures (production ofzymogen for proteases (Docherty and Steiner, 1982; Wendesman, 1989) or prehormones (Julius et al., 1983) or may function as sorting signals (e.g. to the vacuoles in the case of carboxypeptidase Y) to increase the efficiency of secretion of small peptides such as α-factor (Bussey, 1988) or to promote the correct folding of the protein (Ikemura et al., 1987; Ikemura and Inouye, 1988; Silén et al., 1988; Silén and Agard, 1989; Stoller and Shields, 1989).

Although several reports have dealt with the role of proregions in the secretion of heterologous proteins by yeast (Brake et al., 1984; Ernst, 1988; Franke et al., 1988; Heslot et al., 1990), no analysis of the role of these regions in the extracellular secretion of their cognate protein has been reported so far. To investigate the role of a specific proregion we have used AEP which is secreted by the yeast Y. lipolytica in quantities that can reach 1-2 g/liter under optimal conditions (Tobe et al., 1976; Ogrydziak et al., 1977). The XPR2 gene coding for the AEP has been cloned in several laboratories (Davidow et al., 1987; Matoba et al., 1988; Nicaud et al., 1989b). The DNA sequence revealed that it may code for a preproenzyme precursor of 46,903 Da. Following the putative signal sequence there is a stretch of X-Ala or X-Pro sequences that are possible substrates for a dipeptidyl aminopeptidase. This is followed by a proregion that includes a glycosylation site (Asn-225) and two Lys-Arg sequences (positions 59-60 and 156-157) that are possible substrates for a KEX2-like endoprotease. The mature protein starts at amino acid 158 and has a molecular size of 30,524 Da.

Ogrydziak and collaborators (Matoba and Ogrydziak, 1989; Matoba et al., 1988) have shown that the AEP precursor undergoes a complex series of maturation steps. In the following section precursor size will be named according to Ogrydziak and co-workers (Matoba and Ogrydziak, 1989; Matoba et al., 1988). A glycosylated (pre)proprecursor polypeptide of 55 kDa generates an inactive proprecursor of 52 kDa after cleavage of the signal sequence and of the X-Ala/X-Pro sequences.

* This work was supported by grants from the Institut National de la Recherche Agronomique, Centre National de la Recherche Scientifique, and from Pfizer Inc. (Groton, CT). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Recipient of a fellowship from the Ministère de la Recherche et Technologie. To whom correspondence should be sent. Tel.: 1 30 54 45 10; Fax: 1 30 54 56 90.

‡ Supported by a Spanish-French exchange fellowship Ministerio de Educacion y Ciencia/Ministere de la Recherche et Technologie.

§ The abbreviations used are: AEP, alkaline extracellular protease; ER, endoplasmic reticulum; bp, base pair(s); SKM, skim milk plate; SDS, sodium dodecyl sulfate; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
This glycosylated precursor may be secreted in the culture medium by an xpr6 mutant defective in KEX2-like activity (Matoba and Ogrydziak, 1989). This secreted polypeptide consists of a mixture of three precursors starting at Ala3', Val19, and Glu44, confirming that processing involves a dipetidyl aminopeptidase. Experiments with tunicamycin and endoglycosidase H-treated cells showed that both the 55- and the 52-kDa precursors contain about 2 kDa of N-linked oligosaccharide. Further processing generates 44- and 36-kDa precursors and the mature 32-kDa protease. The preproregion can thus be divided into three parts: pro, proII, and proIII, with putative cleavage sites at Lys-Arg20 (pro-proII), Lys192 or Lys20 (proII-proIII), and Lys-Arg27 (proII-mature), respectively. Finally, Ogrydziak and collaborators (Matoba et al., 1988) have proposed the following precursor-product relationship: 55 kDa → 52 kDa → 44 kDa → 36 kDa → 32 kDa. It remains unclear whether the 44- and 36-kDa precursors are part of the main processing pathway and whether signal peptide cleavage occurs early in the ER or later in the secretory pathway (Matoba et al., 1988).

We have shown that the AEP preproregion is not absolutely necessary for extracellular secretion of heterologous proteins. Indeed, strains carrying the interteron-α gene coding for the porcine interferon-α fused to XPR2 after the signal sequence or the pro site of AEP are able to secrete antiviral activity into the growth medium (Heslot et al., 1990). Similar results were obtained with prochymosin fusions (Franke et al., 1988).

In this article we report the effects of deletions and point mutations on the preprosequence and the mature part of the prosequence. We have shown that in addition to a zymogen function, the complete preproregion is required for AEP secretion and that secretion at 28 °C depends on the glycosylation of the precursor. We also confirm that AEP processing corresponds to the precursor-product relationship 55 kDa → 52 kDa → 32 kDa and that the 55-kDa precursor corresponds to the precursor from which the signal sequence has been cleaved.

**MATERIALS AND METHODS**

**Enzymes—**All restriction enzymes were purchased from Appligene. The Klenow fragment was purchased from Amersham Corp., and the Klenov fragment of the DNA polymerase I and T4 DNA ligase were purchased from Boehringer Mannheim.

**Strains, Plasmids, and Media—**Strains and plasmids used are listed in Table I. *Escherichia coli* strain TG1 was used for oligonucleotide mutagenesis and for plasmid DNA propagation. *Y. lipolytica* asexic strains J12, which carries the wild-type *XPR2* gene, and JM53, in which *XPR2* is disrupted, were used as recipients for the different mutated *XPR2* constructs. Restriction maps of the chromosome around the *XPR2* locus are shown in Fig. 1B. The *E. coli* vectors used for mutagenesis were constructed by inserting a 18.8-kilobase Clal/XbaI fragment from pINA152 (Nicaud et al., 1989b) spanning the promoter and the preprosequence of the *XPR2* gene into the Bluescript vectors KS+ or KS- (Stratagene) resulting in plasmid pINA310 (see Fig. 1A). The Y. lipolytica vector pINA310 includes the following features: (i) a Nhel or (MluI)/XbaI cassette containing the promoter and the preproregion of the *XPR2* gene that can be exchanged with the *E. coli* plasmids used for mutagenesis; (ii) the region coding for the mature AEP which can also be excised as an XbaI/Clal fragment for mutagenesis; (iii) unique Mbol or Nhel restriction sites in the *XPR2* promoter region used to target the plasmid to the *XPR2* locus in the chromosome; and (iv) the marker gene LEU2 from Y. lipolytica which can be excised using EcoRI. Plasmid pINA302 (M. Beckerich, this laboratory), used for the in vitro experiments, contains the *XPR2* gene placed under the control of the phage SP6 promoter. In this plasmid, the SP6 promoter of pSP64 (Boehringer Mannheim) is fused through the (destroyed) FseI site to the 5'-noncoding sequence of AEP 13 bp upstream from the ATG. The derivative plasmids pINA467, pINA468, pINA469, and pINA472 containing Del1, Del2, Del3, and DelG, respectively (see “Oligonucleotide Mutagenesis”), were constructed by three-way ligation using the EcoRI/Sfl and Sfl/BglII fragments from pINA392 (containing the SP6 promoter) and the BglII/EcoRI fragment from pINA310 derivative containing the mutations Del1, Del2, Del3, and DelG.

YNB minimal medium and the inducing medium YPD were described previously (Nicaud et al., 1989b). Skim milk plates (SKM) were prepared according to Ogrydziak and Mortimer (1977). Media used for growing *E. coli* were prepared as described in Miller (1972).

**Oligonucleotide Mutagenesis—**The oligonucleotides used for mutagenesis (Fig. 2A) were synthesized using the solid phase phosphotriester method (Eftamou et al., 1983) with a Cyclone DNA synthesizer (Stratagene) and were purified by high pressure liquid chromatography.

Site-directed deletions or other mutations were constructed by the primer extension method of Carter et al. (1985) with minor modifications. Single-stranded matrix DNA was obtained by infection of *E. coli*, strain TG1 containing plasmid pINA302 with λ ACT by the DNA polymerase I (Klenow fragment) at 37 °C for 90 min in the presence of ligase. The hybrid molecules were used to transform TG1 cells. However, when oligonucleotides a or b were used, hybrid molecules were digested with BglII restriction enzyme prior to transformation. Screening of transformants was done either by gel analysis of plasmid DNA (as the creation of restriction sites, by direct selection) by colony hybridization to EcoRV/XbaI fragment, or by colony hybridization with [32P]-labeled oligonucleotide ([γ-32P]ATP, 5,000 mCi/mmol, Amersham Corp.) (Davis et al., 1980). The deletions or mutations were confirmed by sequencing the preproregion from the XbaI site to the ATG codon according to Ganger et al. (1977). The mutagenized cassette was then introduced into the Y. lipolytica vector pINA310 by replacing either the Nhel/XbaI or Mul/XbaI fragment by the mutated fragment.

The resulting plasmids containing deletions or mutations are listed in Table I. Their corresponding fusions sites or amino acid mutations are shown in Fig. 2B, and the encoded proteins are represented schematically in Fig. 3.

Oligomer a was used to delete the entire preproregion and create the Del2 deletion which fused Pro12 (the last amino acid of the X-Ala/X-Pro segment) to Ala24 (first amino acid of the mature form). The resulting plasmid was called pINA310-2. During this mutagenesis, we fortuitously obtained a deletion of the proII-proIII region and an insertion of 14 amino acids (Del3 deletion) which resulted in plasmid pINA310-3.

Oligomer b was used to delete the proII-proIII region (Del3 mutation) which fuses mature AEP (Ala24) directly after the first putative KEX2 site (Lys-Arg4). The resulting plasmid was called pINA310-3.

Oligomer c was used to destroy the glycosylation site in the preproregion by replacing the threonine with a valine (DelG mutation). The resulting plasmid was called pINA317.

Oligomer d was used to inactivate AEP by the addition of four arginines in a region located to the active site. For this, the plasmid pINA310 was linearized by cleavage with BamHI. Ligation was performed in the presence of the phosphorylated oligomer d. Plasmids were cut with BamHI prior to transformation to avoid ligation without linker. The presence of the linker was checked by digesting with the restricted endonuclease PstI. The resulting mutation (1) was sequenced using oligomer c, which corresponds to the sequence near the PstI site from bp 1045 to 1063, the resulting plasmid was called pINA320.

**DNA Preparation—**Plasmid DNA from *E. coli* grown in LB medium was prepared by the Holmes and Cagley (1981) procedure. Chromosomal DNA from *Y. lipolytica* was prepared according to Hoffman and Winston (1987). DNA fragments were separated by electrophoresis in agarose gels and purified using a GeneClean kit (Ozyme).

**Transformation—**Transformation of *E. coli* was performed as described by Dugert and Ethlin (1979). *Y. lipolytica* integrative transformation was performed as described previously (Galliard and Ribet, 1987). Plasmid DNA was cut with Nhel restriction endonuclease before transformation to target integration at the *XPR2* locus. To check the chromosomal structure of the *XPR2* locus, we prepared chromosomal DNA from the *XPR2* fragment by gel electrophoresis, transferred them to Hybond N nylon (Amersham Corp.), and hybridized them to the 1.1-kilobase EcoRV fragment from pINA310 according to Davis et al. (1980).
Protein Analysis—Precultures grown in the noninducing, selective medium YNB were used to inoculate 20 ml of the inducing medium YPDm (Nicaud et al., 1989a) at an initial density of 0.1 OD_{600} in 250-ml flasks. Cells were grown under aeration at 28 °C, and samples were taken in the late exponential phase between 24 and 32 h after inoculation at a density of 15-20 units (OD_{600}). Cells and supernatant fractions were separated by centrifugation at 12,000 × g for 10 min at 4 °C. Cells were washed twice with cold homogenization buffer (50 mM Tris-HCl, pH 6.8, 1 mM MgCl_2, 1 mM CaCl_2), resuspended at the original cell density in this buffer together with 0.5% of acid-washed glass beads (0.45 mm), and disrupted using a Braun homogenizer. Samples were assayed for AEP activity or were analyzed for protein content after treatment with phenylmethylsulfonyl fluoride (2 mM) and aprotinin (0.1%). All protein samples were denatured in an equal volume of 2 × sample buffer (100 mM Tris-HCl, pH 6.8, 2% 2-mercaptoethanol, 4% SDS, 20% glycerol, 0.01% bromphenol blue) for 5 min at 100 °C. Insoluble material was discarded by centrifugation for 5 min at 12,000 × g at 4 °C. For partial fractionation studies, disrupted cells were separated into soluble and insoluble (membrane) fractions by centrifugation at 14,000 × g for 30 min prior to heating at 100 °C with sample buffer.

Proteins were separated in 12% polyacrylamide gels according to Laemmli (1970) and blotted on nitrocellulose filters (Burnette, 1981). Samples loaded onto the gels contained material equivalent to 0.625 μg of protein (see Table 1). The gels were stained with Coomassie blue. After destaining, the gels were dried and exposed to X-ray films (Kodak X-OMAT). Films were scanned with a densitometer (Gelman Instrument Co.), and the protein profiles were quantitated with an densitometer (Gelman Instrument Co.). Films were scanned with a densitometer (Gelman Instrument Co.), and the protein profiles were quantitated with an densitometer (Gelman Instrument Co.).

In Vitro Transcription and Translation—Plasmid pINA392 and its derivatives were linearized downstream from the XPR2 gene by digestion with the restriction endonuclease EcoRI. One microgram of mercaptoethanol, under "Protein Analysis," and tunicamycin (10 μg/ml, Sigma) was added 5 h before culture samples were withdrawn. Protein deglycosylation was carried out with 1 milliunit of endoglycosidase H (Genzyme Inc.) and the level of AEP production were identical in strain JM12 and in JM23WT (JM23 transformed with pINA310) (data not shown). Since we expected that some of the constructs would have a trans effect on the wild-type protease itself, these mutations were studied in both Xpr^+ (JM12) and Xpr^- (JM23) backgrounds (see Table 1). Strain JM23 did not produce AEP but did so once transformed with a plasmid carrying a functional XPR2 gene such as pINA310. In order to compare AEP secretion and production, we have verified that the regulation and the level of AEP production were identical in strain JM12 and in JM23WT (JM23 transformed with pINA310) (data not shown).

Construction of Large Deletions Affecting the Proregion of AEP—For our studies on the role of the proregion, we constructed several deletions in the corresponding region of XPR2. In the derivative designated Del1, the region between Lys-Arg and Ala was deleted. In Del2, the complete proregion is absent, but the X-Ala/X-Pro stretch that may separate the pre- and proregion is retained. Del2 also retains 18 amino acids downstream of the putative signal peptide processing site Ala (Matoba et al., 1988).

TABLE I

| Designation | Relevant genotype or description | Source or ref. |
|-------------|---------------------------------|---------------|
| JM12        | MatA, leu2-35, his3-12, ura3-18, XPR2 | Nicaud et al. (1989a) |
| JM23        | Strain JM12 disrupted for XPR2 (xpr2::LY5S) | Nicaud et al. (1989a) |
| DO613       | MatA, ade1, xpr-13 | Matoba et al. (1988) |

Plasmids

| Designation | Vector containing the Y. lipolytica XPR2 and URA3 genes | Nicaud et al. (1989a) |
|-------------|---------------------------------------------------------|---------------------|
| pINA303     | Vector containing Clai-Xcm1 fragment carrying XPR2 promoter and proregion | This work          |
| pINA310     | Plasmid carrying the XPR2 gene and the LEU2 gene | This work          |
| pINA343     | Vector containing the Y. lipolytica XPR2 and LEU2 genes | S. Blanchin, unpublished data |
| pINA392     | Plasmid carrying the XPR2 gene under the control of the Sp6 promoter | J. M. Beckerich, unpublished data |
| pINA310-1   | Derivative of plasmid pINA310 carrying deletion 1 | This work          |
| pINA310-2   | Derivative of plasmid pINA310 carrying deletion 2 | This work          |
| pINA310-3   | Derivative of plasmid pINA310 carrying deletion 3 | This work          |
| pINA317     | Derivative of plasmid pINA310 carrying a mutated glycosylation site | This work          |
| pINA320     | Derivative of plasmid pINA310 carrying the linker 1 | This work          |
| pINA467     | Derivative of plasmid pINA392 carrying deletion 1 | This work          |
| pINA468     | Derivative of plasmid pINA392 carrying deletion 2 | This work          |
| pINA469     | Derivative of plasmid pINA392 carrying deletion 3 | This work          |
| pINA472     | Derivative of plasmid pINA392 carrying a mutated glycosylation site | This work          |

V. Protease

Alkaline Extracellular Protease—We have reported previously on the cloning of the XPR2 gene coding for AEP. To study the role of the proregion of AEP we developed plasmids pINA303 for mutagenesis and pINA310 to introduce the mutation into the Y. lipolytica chromosome (see "Materials and Methods").

Since we expected that some of the constructs would have a trans effect on the wild-type protease itself, these mutations were studied in both Xpr^+ (JM12) and Xpr^- (JM23) backgrounds (see Table 1). Strain JM23 did not produce AEP but did so once transformed with a plasmid carrying a functional XPR2 gene such as pINA310. In order to compare AEP secretion and production, we have verified that the regulation and the level of AEP production were identical in strain JM12 and in JM23WT (JM23 transformed with pINA310) (data not shown).

RESULTS

Synthesis of Alkaline Extracellular Protease—For our studies on the role of the proregion, we constructed several deletions in the corresponding region of XPR2. In the derivative designated Del1, the region between Lys-Arg and Ala was deleted. In Del2, the complete proregion is absent, but the X-Ala/X-Pro stretch that may separate the pre- and proregion is retained. Del2 also retains 18 amino acids downstream of the putative signal peptide processing site Ala (Matoba et al., 1988).
Role of Prosequence in Secretion of Yeast Protease

A.

Fig. 1. A, restriction maps of plasmids pINA310, pINA303, and pINA392. B, chromosomal restriction maps of the XPR2 locus of the Xpr+ strain JM12 and of the disrupted Xpr' strain JM23. The abbreviations used are: B, BamHI; Bg, BglII; C, ClaI; ClaI (Klen), ClaI site filled in with Klenow polymerase; E, EcoRI; E., EcoRV; M, MluI; N, NheI; P, PstI; X, XbaI; kb, kilobases.
Role of Prosequence in Secretion of Yeast Protease

FIG. 2. A, DNA sequence of the synthetic oligonucleotides used for site-specific mutagenesis, linker insertion, and sequence analysis. B, amino acid sequence of the fusion sites in the deletions or mutation constructs. 1, plINA310-1 (fusion pre-proIIIAEP); 2, plINA310-2 (fusion pre-AEP); 3, plINA310-3 (fusion proproI-AEP); 4, plINA317, mutagenesis of the glycosylation site in the proII region; 5, plINA320, linker insertion in the region coding of the mature AEP. Numbers refer to amino acid positions in wild-type AEP (Davidow et al., 1987).

al., 1988) such that cleavage should not be affected. During these experiments we obtained a third clone corresponding to a deletion in the wild-type XPRB gene. In all cases we obtained at high frequency. In both strains were obtained at high frequency. The proregion is symbolized by an open rectangle divided in three parts (proI, proII, and proIII) according to Matoba et al., 1987). Numbers are indicated in bold capital letters. The abbreviations used are: KR, Lys-Arg sequence; AA, amino acid; B, BamHI restriction site. The exact amino acid sequences of the fusion sites are shown in Fig. 2.
Role of Prosequence in Secretion of Yeast Protease

JM23WT) but appeared in strains such as JM12WT, which carries two copies of XPR2. JM12 also produced twice the normal level of AEP.

Mutation Affecting the Glycosylation Site in the ProII Region and Its Effect on AEP Secretion—Glycosylation is often important for protein secretion. For example, tunicamycin-treated cells are impaired in the secretion of a factor (Julius et al., 1984), invertase (Ferro-Novick et al., 1984), and acid phosphatase (Mizunaga et al., 1988). In some cases, inhibition was reported to be temperature dependent, being more pronounced at 30 °C than at 20 or 25 °C (Mizunaga et al., 1988; Ferro-Novick et al., 1984). To assess if the glycosylation site located in the proII region is important for AEP secretion, we destroyed the Asn-X-Thr recognition site by oligomutagenesis which changed the ACT codon coding for threonine to GTC coding for valine (see Figs. 2B and 3). This glycosylation-minus mutation (DelG) was introduced into Y. lipolytica strain JM23 using pINA317, and the transformants were tested on SKM plates at both 18 and 28 °C. This latter temperature is close to the upper limit for Y. lipolytica growth. All transformants produced clear hydrolysis halos on SKM at 18 °C, whereas no halos were observed at 28 °C. This may have been because strains producing DelG either synthesized an inactive protease due to misfolding induced by the DelG mutation or were unable to secrete AEP. To distinguish between these two possibilities we examined the accumulation of AEP by the strain carrying the DelG mutation. A strain producing a thermosensitive AEP encoded by pINA320, which has a linker insertion (L1) in XPR2 close to the region encoding the active site of the enzyme2 (see “Materials and Methods”), was used as a control in these studies. Precultures were grown at 18 °C and then induced at 18 or 28 °C. Culture samples were collected between 24 and 32 h when grown at 28 °C or between 30 and 38 h when grown at 18 °C. Supernatants and cell fractions were used to determine AEP activities and for protein analysis by immunoblotting.

As shown in Fig. 5A, identical levels of extracellular mature AEP were produced at 18 °C by the three strains (wild type, DelG, and L1). AEP produced by strain L1 migrated slightly more slowly than normal AEP, possibly because two of the four amino acids added by the linker L1 (Ser-Arg-Ser-Arg) are positively charged. Strains carrying DelG and L1 produced lower levels of extracellular mature AEP at 28 °C than did the wild-type strain. For strain L1 the lower level of extracellular mature AEP was due to an instability of the protein.

\*E. Fabre, J.-M. Nicaud, M. C. Lopez, and C. Gaillardin, unpublished results.

Fig. 4. Accumulation of AEP precursors in deletion mutants. Immunoblot analysis of proteins produced by wild-type (WT) and the deletion mutants in strain JM23 (A, Xpr' strain) and strain JM12 (B, Xpr' strain). Induction of JM23 and JM12 transformants was performed in YPDm medium. Proteins from supernatant (S) and cell extracts (C) were prepared as described under “Materials and Methods” and separated by SDS-polyacrylamide gel electrophoresis on a 12% gel, transferred to nitrocellulose, and developed with anti-AEP antibodies. The positions of the molecular size standards (in kDa) are indicated.

Fig. 5. Temperature effects on the accumulation of AEP precursor in AEP mutants. Immunoblot analysis of proteins produced by JM23 transformants containing wild-type (WT, DelG, and L1) constructs induced at 18 or 28 °C. Protein from supernatant (A) and cell extracts (B) were isolated from cells grown at 28 or at 18 °C and were separated on a 12% gel.
Indeed L1 and wild-type AEP present similar half-lives at 18 °C, but L1 is degraded rapidly at 28 °C (data not shown). Cells grown at 18 °C contained mainly the 32-kDa polypeptide and small amounts of a 55-kDa polypeptide for wild-type and L1 strains or a 53-kDa polypeptide for strain DelG. All three strains were observed occasionally to contain small amounts of two other proteins (44 and 36 kDa, not visible on Fig. 5B). The patterns of intracellular proteins produced by the wild-type strain and L1 were identical irrespective of the growth temperature. In contrast, the DelG mutant contained large amounts of a 53-kDa polypeptide precursor (which also fractionates with membranes; see "Materials and Methods") when grown at 28 °C. The appearance of this precursor correlates with the decrease in the amounts of intracellular and extracellular mature AEP. These cells also contained increased amounts of immunoreactive material that may have resulted from degradation of accumulated precursors.

Characterization of AEP Produced by the Various Mutants—The molecular masses of the precursors that result from the translation of Del1, Del2, Del3, and DelG XPR2 derivatives were calculated to be 38,099, 33,620, 36,541, and 46,901 Da, respectively. Signal sequence cleavage after Ala15 would lower these figures by 1.55 kDa; further diaminopeptidase processing would lower them by an additional 1.55 kDa. The observed values for the mutated precursors, 40, 34, 38, and 53 kDa (see preceding sections), are all higher than predicted for precursors having undergone signal sequence and diaminopeptidase processing (see also Matoba et al., 1988). Although there is no potential glycosylation site remaining in the proregion of the various constructs (deleted for Del1, Del2, Del3, or mutated for DelG), there are two such sites (Asn205 and Asn239) in the mature part of the protein. Neither of these sites is normally modified in the wild-type protein. Since the precursors accumulated in the cells, they could misfold in such a way that these glycosylation sites would be glycosylated. We therefore compared the electrophoretic mobility of precursors accumulated in JM12 and JM12 transformants grown in the presence or absence of tunicamycin. The Xpr2 precursor was used as an internal control. As shown in Fig. 6, the accumulated mutant precursors had the same electrophoretic mobility in both cases whereas the wild-type (glycosylated) 55-kDa precursor was converted to a 53-kDa protein. Similar results were obtained when cell extracts were treated with endoglycosidase H (data not shown), indicating that Del1, Del2, Del3, and DelG polypeptides were not glycosylated. Sometimes we observed a faint band above the accumulated precursors in the presence of tunicamycin (see Fig. 6) which is not observed after endoglycosidase H treatment (data not shown). This may result from tunicamycin-dependent inhibition of signal peptidase.

Because higher mobility could not be explained by glycosylation, we compared the migration on acrylamide gels of in vivo products and polypeptides produced in vitro. For the latter, we used pINA392 in which XPR2 is under the control of SP6 promoter and constructed derivatives containing the Del1, Del2, Del3, and DelG mutations. Fig. 7A shows that the precursor produced by strain JM12 containing Del2 migrated faster than the in vitro product, suggesting that the signal

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**Fig. 6.** Studies of glycosylation of AEP precursors produced by the various constructs. Immunoblot analysis of proteins produced by JM12 transformants containing wild-type (WT), Del1, Del2, and Del3 constructs grown in the absence (−) or presence (+) of tunicamycin. Proteins were separated on a 12% gel.

**Fig. 7.** Identification of accumulated AEP precursors. A, comparison of in vivo intracellular accumulated protein (uv) produced by JM12WT (WT), treated (+) or not (−) with tunicamycin, and JM12 transformant containing Del2 (Del2, uv) with their in vitro counterparts (vt). Proteins were separated on a 12% gel. B, JM12WT in vivo precursor and in vitro product from A were resolved on a 7.5% gel. C, comparison of electrophoretic mobility of precursors produced by strain JM12 containing wild-type and JM23 containing DelG, with precursors produced by an xpr6 mutant. Proteins were separated on a 7.5% gel. WT, proteins from JM12WT grown without (−) or with (+) tunicamycin (exposed 1 day); Del2, proteins from JM23DelG in vivo (uv) and its in vitro counterpart (vt) xpr6, xpr6-secreted precursor before (−) or after (+) treatment with endoglycosidase H (exposed 7 days). Proteins were transferred to nitrocellulose, developed with rabbit antibodies, and detected with 35S-labeled donkey anti-rabbit antibodies.

**Fig. 8.** Protease sensitivity of AEP precursors in deletion mutants. Immuno blot analysis of proteins produced by wild-type (WT) strain JM12 and by the deletion mutants in strain JM23. Membranes were prepared as described under "Materials and Methods." They were treated with proteinase K (0.5 mg/ml) (Pk) in the absence (−) or presence (+) of Triton X-100 (0.1%) (Tx). Proteins were separated by SDS-polyacrylamide gel electrophoresis on a 10% gel, transferred to nitrocellulose, and developed with anti-AEP polyclonal antibodies.
sequence was indeed cleaved. Similar results were obtained with Del1 and Del3 (data not shown). The abnormally high apparent molecular masses of these precursors may thus result from the presence of the 10 X-Ala/X-Pro sequences (Matoba et al., 1988; Matoba and Ogrydziak, 1989).

To identify the precursor accumulated in the DelG construct at 28 °C we compared the electrophoretic mobilities of the in vivo DelG product, the in vitro translated DelG polypeptide, the wild-type 55-kDa precursor, and the product secreted by an xpr6 mutant. The in vitro product corresponds to the complete precursor, i.e. pre-(X-Ala/X-Pro)10-pro-AEP. The 55-kDa precursor may be the glycosylated form of this precursor or of (X-Ala/X-Pro)10-pro-AEP (Matoba et al., 1988). Glycosylated pro-AEP is secreted by the xpr6 mutant (Matoto and Ogrydziak, 1989). The corresponding deglycosylated form was obtained by treatment of protein from the supernatant from a culture of this strain with endoglycosidase H. Fig. 7B shows that the deglycosylated 55-kDa wild-type precursor was smaller than the wild-type precursor synthesized in vitro. Similar results were obtained with the DelG construct as shown in Fig. 7C; the DelG protein, which has the same electrophoretic mobility as the deglycosylated 55-kDa wild-type precursor, was smaller than the DelG product synthesized in vitro and about 3 kDa larger than the 52-kDa xpr6 deglycosylated precursor. This suggests strongly that DelG and the 55-kDa wild-type precursor have undergone signal peptide cleavage but not diaminopeptidase processing.

Localization of AEP Produced by the Various Mutants—We have shown that signal sequence cleavage occurred in the AEP derivatives. However, we could not determine if the main part of the protein was still in the cytoplasm or was fully translocated into the ER lumen. As the deletions did not fortuitously generate a new segment of hydrophobic amino acids which could function as a stop transfer signal, we predicted that the mutated AEP precursors were fully translocated to the ER lumen. To confirm this hypothesis, we prepared membrane fractions as described under "Materials and Methods" and tested the protease sensitivity of these precursors. As shown in Fig. 8, all Del precursors as well as the 55-kDa wild-type precursor were protease resistant in the absence of detergent and became sensitive in its presence. Protease K activity was checked by its action on the total protein pattern (Coomassie Blue staining; data not shown). We observed that mature AEP is resistant to proteasome K treatment, which may be due to the fact that AEP is a serum protease, as is proteasome K. These results confirm that Del precursors have entered the secretory pathway.

DISCUSSION

In this study, we analyzed the secretion of alkaline extracellular protease by the yeast Y. lipolytica. We have identified the precursors and have shown that mutations in the proregion affect secretion drastically.

Secretion of AEP from Y. lipolytica is a rapid process that involves the synthesis of a pre-(X-Ala/X-Pro)10-pro-AEP polypeptide. The first precursor seen by pulse labeling is a 55-kDa polypeptide that contains about 2 kilodaltons of N-linked carbohydrate. According to Matoba et al. (1988) and

3 Strain D0613 (xpr6 mutant) was expected to produce an unglycosylated 52-kDa polypeptide when grown at 18 °C in the presence of tunicamycin and, like the DelG mutant, to be unable to secrete AEP at 28 °C. In fact, growth of this strain at 18 °C with or without tunicamycin resulted in the production of mainly 32- and 36-kDa proteins, indicating that the xpr6 mutant is either leaky or temperature sensitive. Thus, the only method available to us to produce the unglycosylated AEP precursor manufactured by the xpr6 mutant was that described above.

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AEP to reach the surface of these cells may be due to aggre-
gation or misfolding of the precursors, resulting in their reco-
ognition by a protein such as immunoglobulin heavy chain
binding protein (Deshaias et al., 1988), which prevents their
further transport through the secretory pathway. This mis-
folding or aggregation is not temperature dependent because,
except for DelG, it occurred at both 18 and 28 °C. The increase
in the amount of the 55-kDa polypeptide in JM12 transform-
ants containing the deletion constructs or when we increased
the XPR2 copy number (Fig. 4) suggests that there is a satu-
rable step at this stage. However, the perturbation is not
important enough to generate growth or morphological
changes as observed in S. cerevisiae with the sec mutants
(Novick et al., 1981) or when overproducing acid phosphatase
(James et al., 1988).

Glycosylation affects intracellular transport and AEP pro-
duction in a temperature-dependent manner. Our results in-
dicate that N-glycosylation of pro-AEP is essential for ER to
Golgi transport and/or proteolytic processing at 28 °C (Fig.
5). Indeed, the 53-kDa AEP precursor, which accumulates in
large amounts in the DelG, appeared to be unable to migrate
further through the secretion pathway at 28 °C. Such temper-
ature sensitivity was also observed for the processing of the
prosubtilisin (Ikemura et al., 1987). Similarly, Mizunaga et
al. (1988) showed that when S. cerevisiae is grown at low tem-
perature in the presence of tunicamycin, the active nongly-
cosylated form of acid phosphatase is secreted. These results
suggest that lower temperature may (i) improve the confor-
mation of secretory protein precursors such as pro-AEP (in-
creasing the time span for a correct folding of the precursor,
(ii) inhibit the interaction with an ER luminal protein such as immunog-
ulbin heavy chain binding protein; or (iii) promote an interac-
tion with a secretion factor (increasing the time for a correct
folding of the precursor, suggesting for invertase (Shulke and
Shmid, 1988) or for

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