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Selective Protein Degradation in the Yeast Exocytic Pathway

Ardythe A. McCracken and Kristina B. Kruse

Department of Biology, University of Nevada, Reno, Reno, Nevada 89557

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Protein degradation in the exocytic pathway was studied in *Saccharomyces cerevisiae* using human alpha-1-protease inhibitor (A1Pi) as a reporter molecule. Yeast cells transformed with A1Pi cDNA genes synthesized A1Pi that entered the secretion pathway and accumulated in the endoplasmic reticulum (ER). Cells expressing A1PiM (wild-type) accumulated about 10-fold more A1Pi than cells expressing A1PiZ (secretion defective variant). Analyses of A1Pi mRNA indicated that the low level of A1PiZ relative to A1PiM was not the result of differential gene transcription. Pulse-chase A1Pi radiolabeling showed that A1PiM and A1PiZ were degraded at different rates and suggested a rapid specific turnover of newly synthesized A1PiZ in the ER. Accumulated A1Pi was degraded at comparable rates in both wild-type cells and cells deficient in vacuolar protease activity, indicating that degradation of A1Pi did not occur in the vacuole. Studies to investigate the intracellular location of the degradative process, using temperature-sensitive secretion defective yeast strains, suggested the possibility that degradation occurs not only in the ER but at a second site accessed by vesicle transport. Together, these results demonstrate that a selective protein degradation process operates early in the yeast cell exocytic pathway.

INTRODUCTION

An intracellular protein degradation process has been described that appears to function in an early compartment of the mammalian cell secretion pathway. This process may operate as an essential step in the quality control of newly synthesized proteins, a fundamental control for cellular function. Several studies have shown that this degradative process is selective and is distinct from the well-known lysosomal degradation pathway (for reviews see Bienkowski, 1983; Klausner and Sitia, 1990).

Recent reports have implicated this proteolytic pathway in the degradation of numerous proteins including HMG-coA reductase (Chun *et al.*, 1990; Inoue *et al.*, 1991), variants of alpha-1-proteinase inhibitor (A1Pi) and alpha-2 plasmin inhibitor (Le *et al.*, 1990; Miura and Aoki, 1990), preprosomatostatin-alpha globin fusion protein (Stoller and Shields, 1989), apolipoprotein B (Davis *et al.*, 1990; Sato *et al.*, 1990), truncated forms of ribophorin I (Tsao *et al.*, 1992), fibrinogen B-beta chain (Daniskhfsky *et al.*, 1990), acetylcholinesterase (Rotundo *et al.*, 1989), and several viral proteins (Gabathuler *et al.*, 1990; Navarro *et al.*, 1991; Ulmer and Palade, 1991). Each of these proteins, and those described in the earlier reviews, appears to accumulate in the endoplasmic reticulum (ER). However, lack of transport from the ER is not sufficient to induce degradation, indicating that the degradative process is selective (Bonifacino *et al.*, 1990; Valetti *et al.*, 1991; Tsao *et al.*, 1992). The selectivity of this degradation process is also demonstrated by differences in half-lives of the degraded proteins, which range from 5 min to several hours; and in the case of the alpha chain of the T cell receptor, selectivity is demonstrated by specific amino acid sequence information (Bonifacino *et al.*, 1990).

Two lines of evidence suggest that the intracellular site for this degradative process is the ER. First, evidence that lysosomal enzymes are not involved in this degradation process has been obtained by examining the effect, on this process, of agents that inhibit lysosomal function. Such experiments with the various proteins mentioned above demonstrated that degradation was not inhibited by members of these lysomotropic agents or by protease inhibitors known to be specific for lysosomal hydrolases. Second, on the basis of carbohydrate content and intracellular localization studies with the light and electron microscope, the accumulated substrates do not appear to leave the ER. Together, these data suggest that degradation does not occur in the lysosome but in the ER itself. To further test this hypotheses, degradation has been reconstructed in semi-

permeable cells. Such experiments have shown that vesicular transport from the ER was not required for the initial proteolytic cleavage leading to the degradation of H2a (Wikström and Lodish, 1992) or for the degradation of chimeric integral membrane T cell receptor subunits (Stafford and Bonifacino, 1992). These results support the premise that degradation takes place in the ER. However, a truncated luminal mutant of ribophorin I appears to be degraded by two processes, one in the ER and a second accelerated process that is inhibited by blocking vesicle transport with carbonyl cyanide m-chlorophenylhydrazone and monensin (Tsao et al., 1992). This result suggests that some degradation of this mutant ribophorin takes place in a post-ER pre-Golgi site and thus brings in question the intracellular site for the degradation pathway.

The results from many studies have clearly established the existence of a selective proteolytic system that functions to remove proteins that are not competent for export from the ER. However, many questions regarding the molecular mechanisms of this intracellular protein degradation pathway remain unanswered, in part, because of the absence of knowledge of the identity and function of the responsible proteases. The experiments presented here were stimulated by the desire to elucidate the molecular mechanisms of this process through the identification of the relevant proteases and accessory proteins. Because of the similarities between the yeast and animal cell secretion pathway and the facility with which molecular genetic technology can be applied to yeast to associate a protein with its function (Botstein and Fink, 1988), we chose the yeast system for our studies. We first asked whether or not the yeast cell would demonstrate a selective degradation of proteins in the exocytic pathway. The studies reported here demonstrate that *Saccharomyces cerevisiae*, indeed, has such a protein degradation pathway that is independent of vacuolar proteolysis and that this degradation process may not be restricted to the ER.

MATERIALS AND METHODS

Plasmid Constructs

Human A1Pi genes were subcloned into pBM743, a yeast vector containing a centromere sequence, and into the pYES2.0 vector (Invitrogen, San Diego, CA) containing the 2-μm replication sequence. Cloning was accomplished following standard procedures described in Sambrook *et al.* (1989), by inserting the A1Pi gene sequences into the *Eco*RI-*Sal* I sites of the multiple cloning regions of the vectors. These insertions placed the A1Pi genes in the correct orientation under control of the *Gal1* promoter in both vectors. Both the centromeric and 2-μm containing plasmid vectors carry the URA3 gene for selection of transformants.

Yeast Strains and Cell Culture

All yeast strains were *S. cerevisiae* and were cultured following the procedures of Sherman *et al.* (1986). BC212 cells (Mat alpha ura3-52 leu2-3-112 his3-Δ1 ade2-1) were provided by Dr. Bill Courchesne, University of Nevada, Reno. AB122 (Mat a prc1-407 prb1-1122, pep4-3, leu2 ura3-52, a derivative of BJ2168) (Zubenko *et al.*, 1982) was provided by Dr. Tony Brake, University of California, San Francisco. RSY299 (Mat alpha ura3-1 ade2 his3-11 leu2-3, 112 trp1-1 sec7-1), RSY269 (Mat alpha ura3-52 his 4-619 sec17), and RSY271 (Mat alpha ura3-52 his4-619 sec18-1) were provided by Dr. Randy Schekman, University of California, Berkeley. Cells were transformed following the lithium acetate procedure (Ito *et al.*, 1983), and transformants were selected by growth on synthetic minimal media without uracil. Transformed cells were cultured at 30°C in selective media with 2% glucose as carbon source or were incubated in selective media with 2% galactose to induce expression of A1Pi. Temperature-sensitive mutants were grown at 25°C as permissive temperature and assayed at 37°C for the nonpermissive temperature, except for sec18 cells for which the nonpermissive temperature was 35°C.

Cell Lyses and Fractionation

Cell lysates were prepared by either of two procedures. 1) The cell wall was first removed by incubation at 37°C with 1 mg/ml lyticase (Sigma, St. Louis, MO; L-8012) in lyticase-lyses buffer (40 mM sorbitol, 150 mM potassium acetate, 0.5 mM ethylene glycol-bis(β-aminaethyl ether)-*N,N,N',N'*-tetraacetic acid [EGTA], 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] pH 7.5, 2 mM magnesium chloride, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 0.1 mM L-1-tosylamide-2-phenylethylchloromethyl [TPCK], 1 mM benzamidine hydrochloride, 25 uM pepstatin A). The spheroplasts were then lysed on ice by addition of an equal volume of 2% Triton X-100 (Sigma, St. Louis, MO). Cell lysates were brought to 1 ml at 0.5% Triton X-100 by addition of lyticase-lyses buffer. 2) Lysis of whole cells was accomplished in 0.5% 2-mercaptoethanol, 0.2 M NaOH followed by trichloroacetic acid precipitation and resuspension of protein in 2× RIPA (10 mM tris(hydroxymethyl)aminomethane [TRIS] pH 7.5, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS]). For fractionation experiments, cell samples of 50 OD units were suspended in lyticase spheroplasting buffer (1 M sorbitol, 2 mM EDTA, 0.1% beta-2-mercaptoethanol) at 37°C for 90 min and centrifuged at 500 × *g* for 5 min; the supernatant was collected as the periplasmic fraction. The spheroplasts were washed once; the wash was pooled with the periplasmic fraction and then lysed by freeze thaw in hypotonic buffer (40 mM sorbitol, 150 mM potassium acetate, 20 mM HEPES, 2 mM $MgCl_2$, 5 mM PMSF, 1 mM TPCK, 10 mM benzamidine, and 259 μM Pepstatin A [Sigma]). The lysate was centrifuged, and the supernatant was collected as the cytoplasmic fraction. The membrane pellet was solubilized in 1% Triton X-100.

RNA Isolation and Analyses

Cell cultures were incubated in galactose-containing media for 7 h to induce the expression of A1Pi. Samples of 50 OD units were processed for extraction of whole cell RNA according to the procedures of Sambrook *et al.* (1989). Poly-A RNA was purified from the whole cell RNA using oligo dT (Pharmacia, Piscataway, NJ). Dot blots and Northerns were prepared following standard procedures. A1Pi cDNA isolated from the pBM743/A1PiM vector by *Eco*RI-*Sal* I double restriction digest was denatured by boiling in 60% formamide, quick chilled on ice, and used as control.

Enzyme-linked Immunoadsorbent Assay (ELISA) Detection of A1Pi

Samples and standards were brought to 0.5% Triton X-100 before placing on the ELISA plate. All ELISA were carried out by the sandwich technique as described previously (McCracken and Fishman, 1986) using antibody to human A1Pi (Dako, Carpinteria, CA) at 5 μg/ml to coat the plate and peroxidase-conjugated anti-human A1Pi (Organo Teknika-Cappel, Malvern, PA) diluted 1:1000. Standard curves for quantification were established using purified human A1Pi (Sigma A-9024).

Pulse Chase Protein Radiolabeling

Cells were incubated overnight in selective media free of sulfate and methionine. They were then induced by incubation in media containing galactose but no sulfate or methionine. Cells were concentrated to 5 OD units per ml, and 15–20 μCi/ml ^{35}S-methionine was added for 15–45 min and chased for the indicated durations by washing and addition of 2.4 mg/ml cold methionine. Samples of 10–15 OD units were collected into sodium azide (10 mM) on ice, and all chase samples were concentrated by centrifugation, resuspended in 200 μl lyticase-lyses buffer, and frozen at −20°C. Cells were lysed as described above and immunoprecipitated as previously described (McCracken *et al.*, 1989). The immunoreactive proteins were immunoprecipitated a second time after removal of the antibody-antigen complexes from the staph-A by boiling in 50 μl Laemmli buffer (50 mM Tris, pH 6.8, 2% SDS, 30% glycerol, 5 mM EDTA, 5 mM 2-mercaptoethanol). The twice-immunoreactive proteins were resolved on 10% polyacrylamide gel electrophoresis, observed by fluorography, and quantified by densitometry as previously described (McCracken *et al.*, 1991). Densitometric analysis of the autoradiogram was carried out with a Hoeffer GS300 scanning densitometer (San Francisco, CA) under conditions where the response of the film and the instrument was in the linear range. Computer-assisted integration of the area under the peaks was used to derive a relative area/density unit for each band. For Endoglycosidase-H (Boehringer Mannheim, Indianapolis, IN) treatment, the immunoadsorbed pellet was resuspended in 50 μl of 30 mM NaCitrate, pH 5.0, containing 0.5 mM PMSF, 0.5 μg/ml bovine serum albumin, and 0.02% SDS. One-half of the sample was transferred to a fresh tube and one milliunit of Endoglycosidase-H was added. Both samples were incubated at 37°C for 14–16 h. First order decay curves, generated using Cricket Graph software (Cricket Software, Malvern, PA), were used to determine the half-life of A1Pi.

RESULTS

The results presented here provide information to: 1) establish that a selective degradation process occurs in the exocytic pathway of yeast, 2) show that this form of intracellular protein degradation is distinct from vacuolar proteolysis, and 3) suggest the possibility of multiple sites for A1Pi degradation in the yeast secretion pathway.

A Selective Degradation Process Occurs in the Exocytic Pathway of Yeast

Protein degradation in the yeast exocytic pathway was monitored by following the fate of human A1Pi after expression in various yeast strains. In humans, A1Pi is synthesized primarily in the liver and secreted into the blood, where it serves as the major protease inhibitor. The Z variant of A1Pi (A1PiZ) is associated with a circulatory deficiency of this protein that results from its retention within the ER of hepatocytes. Homozygosity for the A1PiZ allele predisposes individuals to develop pulmonary emphysema and liver disease. Transgenic mice models of A1Pi deficiency, in which several copies of A1PiZ are expressed, develop liver necrosis, and the degree of damage correlates with the amount of A1PiZ accumulation (Dycaico *et al.*, 1988; Carlson *et al.*, 1989). In addition, it appears that much of the A1PiZ synthesized in liver is degraded by a process thought to occur early in the secretion pathway (Le *et al.*, 1990,1992). For these experiments, A1Pi cDNA genes, which encode the natural A1Pi signal sequence, were subcloned into a centromeric yeast plasmid (pBM743) under control of the GAL1 promoter (Johnston and Davis, 1984). Yeast cells transformed with these plasmids can be induced to synthesize A1Pi by growth in galactose. Two forms of the A1Pi cDNA genes were used: A1PiM, the wild-type gene, and A1PiZ, a mutant gene that encodes the secretion defective variant of this protein. When these genes were transiently expressed in COS cells, A1PiM protein was secreted, whereas A1PiZ protein was retained in the ER (McCracken *et al.*, 1989,1991).

Quantitative Analysis of A1Pi Demonstrated that Transformed Cells Expressing A1PiM Accumulate More A1Pi than Those Expressing A1PiZ. The amount of A1Pi in subcellular fractions of transformed cells expressing either A1PiM or A1PiZ was measured by ELISA (Table 1). Most of the A1Pi synthesized was present in the membrane fraction and the periplasmic space (secreted A1Pi), and no A1Pi was detected in the culture medium. Less than 11% of the total A1PiM was present in the cytoplasmic fraction, and much of that may have resulted from vesicle leakage during spheroplast lyses. Therefore, the analyses of A1Pi levels in subcellular fractions demonstrated that A1Pi synthesized in transfected yeast cells entered the secretion pathway.

The apparent subcellular distribution of A1PiZ shown by these results was not consistent with the known behavior of this protein in mammalian cells. For example, these results indicated that nearly 30% of A1PiZ was secreted (periplasm), whereas only 10–15% is secreted from mammalian cells. Subsequent experiments that demonstrated that A1PiZ was rapidly degraded provided an explanation for this discrepancy. That is, because the total amount of A1PiZ synthesized is not available from these data, the percentage of A1PiZ secreted cannot be determined from the values shown in Table 1. However, on the basis of the 3-h half-life of A1PiM, one could assume that during the 6 h induction period the total amount of A1PiM synthesized was twice that detected, i.e., 40 μg. If A1PiZ was synthesized at

Table 1. A1Pi in subcellular fractions of transformed cells

	A1Pi (ng/OD unit)			
Gene/strain	Total	Periplasm	Cytoplasm (% total)	Membranes
A1PiM/AB122	18.87	4.72	1.89 (10)	12.26
A1PiZ/AB122	2.06	0.63	0.32	1.08
A1PiM/BC212	19.80	4.35	2.18 (11)	13.27
A1PiZ/BC212	2.16	0.58	0.34	1.23

Transformed cells grown to log phase in glucose media were shifted to galactose media for 5–7 h. Cell fractions were collected as described in MATERIALS AND METHODS, and A1Pi was quantified by ELISA. No A1Pi was detected in cells transformed with pBM vector without the A1Pi gene inserts. The data represents an average of four experiments.

the same rate as A1PiM, then the amounts secreted would be 0.58/40 = 1.5% for A1PiZ and 4.35/40 = 11% for A1PiM. Although this calculation is for the purpose of demonstration, it is useful to point out that both forms of A1Pi are inefficiently secreted by the transfected yeast cells.

Studies of Carbohydrate Content and Electrophoretic Mobility of A1Pi Indicated Accumulation in the ER. A1Pi is a glycoprotein that receives three carbohydrate chains in the ER. These core carbohydrates are then decorated by outer chain mannoses when the protein is transported through the yeast Golgi (Esmon *et al.*, 1981; Moir and Dumas, 1987). Endoglycosidase-H treatment removes both the outer chain–complexed carbohydrate and core carbohydrates from the polypeptide backbone of yeast glycoproteins. When radiolabeled A1Pi was immunoprecipitated from yeast cell lysates, four bands were observed with the predicted molecular weight and sensitivity to Endo-H expected for the ER forms of A1Pi (Figure 1). Thus, based on the results of Table 1 and Figure 1, we can conclude that A1Pi synthesized by yeast entered the secretion pathway and accumulated in the ER. On some autoradiograms we were able to detect a high molecular weight smear of the heterogeneous outer chain–complexed carbohydrate form (secreted form) of A1PiM, indicating that some A1PiM was transported through the Golgi. This mature form of A1PiM was sensitive to Endo-H and was not observed for A1PiZ.

The Low Level of A1Pi in Cells Expressing A1PiZ Was Not the Result of Differential Gene Expression. The gene sequences for A1PiM and A1PiZ differ by a single nucleotide, and the mRNAs have been shown to be equally competent for translation in a cell-free system (Bathurst, 1984; Verbanac and Heath, 1986). However, a new mutation in the A1PiZ gene or *GAL1* promoter could lead to lowered expression and thus explain the above results. To investigate this possibility, RNA from galactose-induced transformants was analyzed by dot blot and Northern analysis (Figure 2). Both dot blot of whole cell RNA and Northern analysis of purified poly-A RNA demonstrated similar levels of A1Pi specific RNA in cells expressing A1PiM or A1PiZ.

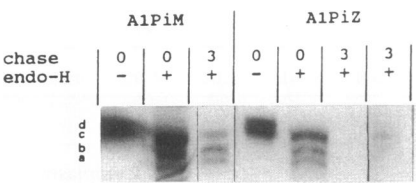

Figure 1. Glycosylated forms of radiolabeled A1Pi from yeast. BC212 cells expressing A1PiM were pulse labeled for 45 min with ^{35}S-methionine, chased for 0 and 3 h in the presences of cold methionine, and one-half of the immunoreactive proteins in each sample was treated with Endoglycosidase-H (+ or −). All samples were processed as described in MATERIALS AND METHODS. The right most lane is an overexposure of the prior lane showing Endo-H–treated A1PiZ at 3 h. a–d indicate forms of A1Pi that differ by amount of carbohydrate: (a) 44 kDa, predicted size for A1Pi without the presequence and without carbohydrate; (b) 47 kDa, presequence-cleaved polypeptide with one core carbohydrate; (c) 50 kDa, presequence-cleaved polypeptide with two carbohydrates; (d) 53 kDa, presequence-cleaved polypeptide with three carbohydrate chains.

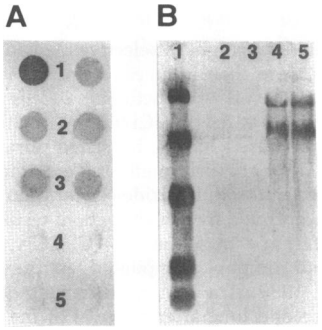

Figure 2. Levels of A1Pi specific RNA from AB122 cells. (A) Dot blot. Row 1, controls of 0.1 and 0.01 pg of linear denatured A1Pi cDNA. Rows 2–5 are 25 μg duplicates of whole cell RNA: row 2, A1PiZ-transformed cells; row 3, A1PiM-transformed cells; row 4, cells transformed with pBM743 vector without gene insert; row 5, untransformed cells. (B) Northern. Lane 1, control restriction digested linear denatured pBM743-A1Pi DNA fragments 3.7, 2.4, 1.3, 0.5, and 0.3 kb. Lanes 2–5, 5 μg poly-A RNA isolated from various strains: lane 2, cells transformed with pBM743 vector without gene insert; lane 3, untransformed cells; lane 4, A1PiZ-transformed cells; lane 5, A1PiM-transformed cells. Both blots were probed with A1Pi cDNA using the BMB genius kit. Similar results were seen for BC212 cells. The two different size A1Pi transcripts may reflect cryptic termination sites in the vector as the A1Pi cDNA insert does not contain a yeast transcription termination site.

Together, these observations that both A1PiM and A1PiZ genes were transcribed at the same level, that both A1PiM and A1PiZ accumulate in the ER, and that ~10-fold more A1PiM than A1PiZ was present following a 5–7-h induction, suggest that a specific degradation of A1PiZ may be occurring in the yeast ER.

Protein Degradation in the Yeast Exocytic Pathway Is Distinct from Vacuolar Proteolysis

As discussed above, transformed yeast cells (BC212) expressing A1PiM accumulate more A1Pi than cells expressing A1PiZ. This same relationship was seen in a yeast strain deficient in vacuolar protease activity (AB122), suggesting that vacuolar proteases were not required for this degradation process (see Table 1). To further study the effect of vacuolar proteases on the degradation of A1Pi, A1Pi was measured during a 10-h induction in both wild-type BC212 and the vacuolar protease deficient strain, AB122. In addition, the fate of A1Pi accumulated during the first 5 h of the induction period was determined in the absence of further synthesis (Figure 3).

Results from these experiments showed a rapid accumulation of A1PiM and a slow minimal accumulation of A1PiZ in both AB122 and BC212 strains (note the different scale on the vertical axis). This result suggested a rapid specific degradation of A1PiZ that was not de-

pendent on vacuolar proteolysis. In addition, when synthesis was terminated by returning the induced cells to media containing glucose instead of galactose, a decrease in the amount of both forms of A1Pi was observed. No A1Pi was detected in the galactose-containing culture media, indicating that A1Pi was being degraded. The half-life of A1PiM in BC212 cells was 3 h and 2.5 h in AB122 cells, whereas the half-life of A1PiZ was 1.5 h in both strains. These data demonstrated that the degradation process does not require the vacuolar proteases.

Degradation of A1PiZ Occurs in the ER and Possibly a Post-ER Compartment

The results of the experiment discussed above showed that both forms of A1Pi were degraded and that A1PiZ was degraded faster than A1PiM. To further study the degradation of A1Pi in yeast, we began pulse-chase protein radiolabeling experiments as described in METHODS AND MATERIALS. The results (Table 2) showed more A1PiM than A1PiZ even at the 0 time point and demonstrated that pools of both A1PiM and A1PiZ protein that had accumulated during the 45 min pulse decreased in amount over the duration of the chase. The half-life of A1PiM determined from these data was about 3 h, whereas the half-life of A1PiZ was 35 min. This differential degradation reflects the selectivity of this process.

A1PiZ Accumulates when Vesicle Transport to the Golgi Is Blocked. To attempt to define the functional intracellular location for A1Pi degradation in yeast and to evaluate the requirement for vesicular transport on the proteolytic process, we began studies of A1Pi expressed in temperature-sensitive secretion mutant strains. The rationale was that a secretion block that inhibited delivery of A1PiZ to the degradative compartment would result in accumulation of this protein. Thus, by blocking the secretion pathway at different steps, it may be possible to identify the site of degradation. The availability of these temperature-sensitive secretion mutants provides an advantage over the use of pharmacological studies to block vesicle transport, because these agents may affect other possibly relevant

Table 2. Pulse-chase A1Pi radiolabeling of BC212 transformants

Chase time (min)	Density units (relative amounts of A1Pi)	
	A1PiM	A1PiZ
0	9842	1604
15	9094	1144
45	8394	717
90	7431	310
150	5577	85
180	4602	67

Transformed cells were radiolabeled and processed as described in MATERIALS AND METHODS. Density units shown here are normalized numbers from four experiments. Half-lives were determined from first order decay curves ($R^2 > 0.98$) generated from this data using Cricket Graph software; A1PiZ $T_{1/2}$ = 35 min, A1PiM $T_{1/2}$ = 3 h.

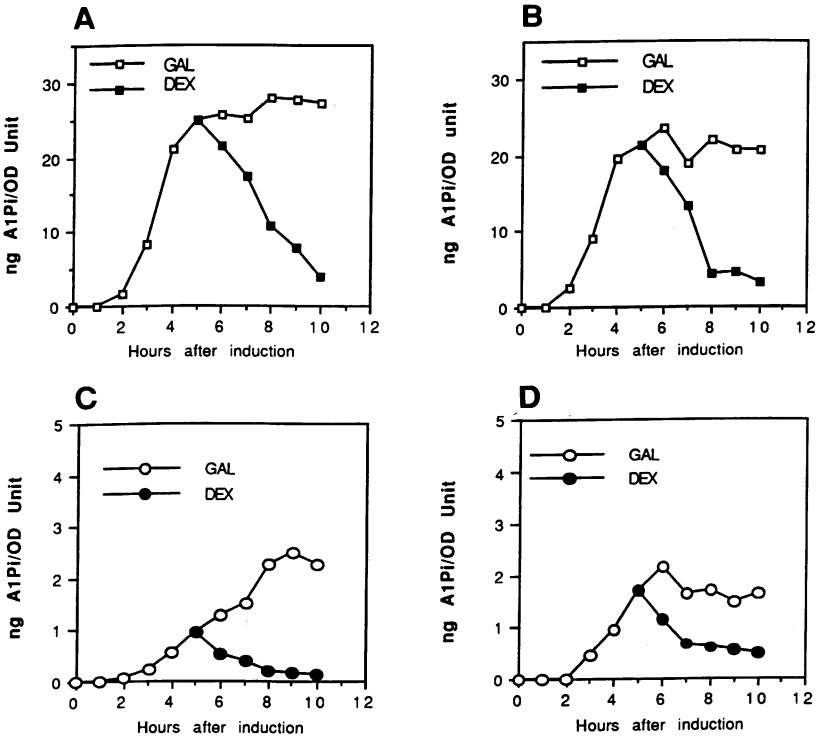

Figure 3. Induction and degradation of A1Pi. Cells grown in glucose were changed to galactose-containing media to induce synthesis of A1Pi. Samples were collected over a 10-h period and assayed by ELISA for A1Pi. At 5 h postinduction, cells were washed, and one-half was resuspended in galactose-containing media whereas the other half was resuspended in glucose-containing media, and both were cultured at 30°C. To eliminate the effect of dilution of protein within a growing population, the levels of A1Pi present in the glucose samples were corrected for growth. (A) BC212 cells transformed with A1PiM. (B) AB122 cells transformed with A1PiM. (C) BC212 cells transformed with A1PiZ. (D) AB122 cells transformed with A1PiZ. Open circles indicate levels of A1Pi for cells cultured in galactose-containing media (induction). Closed circles indicate levels of A1Pi in cells after switch to glucose-containing media (repression). The graphs were generated using Cricket Graph software and the half-life of A1Pi was determined from first order decay curves ($R^2 > 0.960$) generated from these data: BC212/A1PiM $T_{1/2}$ = 3 h, BC212/A1PiZ $T_{1/2}$ = 1.5 h, AB122/A1PiM $T_{1/2}$ = 2.5 h, AB122/A1PiZ $T_{1/2}$ = 1.5 h.

parameters of the degradation process. Results of our studies with three of the sec^{ts} mutants are shown in Table 3. At the nonpermissive temperature, Sec7 cells are defective in transport of secretory protein from the trans-Golgi network into secretory vesicles. Sec18 and Sec17 cells express a temperature-sensitive defect in protein transport from the ER to the Golgi (Novick and Schekman, 1983). Each mutant strain was transformed with A1PiZ and A1PiM, and expression of A1Pi was induced in the transformants at both temperatures. The results showed that when transport of A1Pi was blocked before delivery to the Golgi, there was an increase in accumulation of A1PiZ (see Sec17 and 18, 37/25°C ratio) (Table 3). However, if the ts block was imposed after transport through the Golgi (Sec7), little change was seen in the relative levels of A1Pi. When Wolf and colleagues were studying the essential properties of yeast vacuolar proteases, they observed similar results (Teichert *et al.*, 1989). They reported that the degradation of a mutant prc1-1 carboxypeptidase yscY precursor protein was not altered in the absence of vacuolar proteinases yscA and yscB and showed that this protein had a 30-min half-life in wild-type cells but was stabilized in sec18 cells at 37°C.

DISCUSSION

Together, the data presented here show that most A1Pi synthesized in yeast cells entered the secretion pathway, accumulated in the ER, and was subsequently degraded by a process independent of vacuolar protease activity. Different rates of degradation were observed for A1PiM and A1PiZ that indicated that the proteolytic process was selective. Therefore, the results of this study show that the yeast *S. cerevisiae* have an intracellular proteolytic pathway for the selective degradation of proteins that inappropriately accumulate in the ER.

The differences in the rates of degradation of A1PiM and A1PiZ do not fully account for the difference in the steady-state levels of these proteins, suggesting that there must be other factors involved. The mathematical model that is most often used for determining degradation rates is based on the assumption that protein degradation is a first-order process where the half-life equals $ln\ 2/K_d$ in which K_d is the rate constant for degradation (Schimke, 1970). With this simple model, the level of the protein at steady state (P_L) is determined by the ratio of the rate constant of synthesis (K_s) to the rate constant of degradation (K_d), ($P_L = K_s/K_d$). The application of this model to the analysis of the data in Figure 3 predicts that a factor contributing to the ~10-fold difference in the steady-state level of A1PiZ and A1PiM may be a differential synthesis of these proteins ($K_{sM} = 0.10$, $K_{sZ} = 0.02$). If this is real, it would be an interesting finding because the present knowledge of the mechanisms of translational control in yeast (Hinnebusch and Liebman, 1991) does not include an explanation for differential translation of these two messages that differ only in a single nucleotide within the coding region. However, the approximate sixfold difference in the relative amounts of A1Pi accumulated during a short 45 min pulse (Table 2) is essentially explainable by the approximate 5.3-fold difference in stability measured in these experiments.

A possible explanation for the disparity in steady-state levels of A1Pi could be differential degradation of A1PiZ. The pool of A1PiZ may include two populations: one of extremely unstable nascent molecules with a very rapid degradation rate and one containing molecules that have evaded rapid proteolysis, are somehow more resistant to degradation, and thus, will be degraded at a slower rate. Such an explanation is consistent with the observation that A1PiZ accumulates in inclusion bodies in the ER of liver cells (Bathurst *et al.*, 1984). The

Table 3. Intracellular A1Pi in temperature-sensitive secretion mutants

		A1Pi (ng/OD unit)			
Gene/mutant	ts Sec block	25°C	37°C	37/25°C	Average ratio
A1PiM/Sec 7	Golgi → sec ves	0.92	2.03	2.2	1.9 ± 0.3
A1PiZ/Sec 7		0.25	.48	1.9	1.7 ± 0.2
A1PiM/Sec 18	ER ves → Golgi	5.1	5.6	1.1	1.0 ± 0.1
A1PiZ/Sec 18		0.2	1.22	6.1	6.0 ± 0.5
A1PiM/Sec 17	ER ves → Golgi	3.5	3.5	1.0	1.1 ± 0.2
A1PiZ/Sec 17		0.25	1.2	4.8	5.0 ± 0.3
A1PiM/BC212	Wild-type	12.8	11.8	0.9	1.0 ± 0.2
A1PiZ/BC212		1.4	1.1	0.8	0.9 ± 0.3

Transformed cells grown to log phase in glucose media were shifted to galactose media and incubated at the indicated temperature for 4–5 h. Cells were lysed and A1Pi was quantified by ELISA as described in MATERIALS AND METHODS. Values are from a single experiment except for the average ratios that were calculated from those of four separate experiments.

pool of A1PiM in this model contains one population of relatively stable molecules. Degradation rates were measured using two approaches: pulse chase (Table 2) and induction/repression (Figure 3). Because the pulse chase experiment should yield a pool of A1PiZ that contained more of the nascent molecules (a 45-min pulse) than that of the induction/repression experiment (a 5-h induction), our model of differential degradation would predict that the rate of degradation of A1PiZ would be faster in the pulse chase experiments and that the rate for A1PiM would be similar in both. The half-lives determined for A1Pi in BC212 cells agreed with this prediction (i.e., 35 min for A1PiZ, 3 h for A1PiM in the pulse chase experiment and 90 min for A1PiZ, 3 h for A1PiM in the induction/repression experiment). The differential degradation model would explain the differences in the calculated K_s values and half-lives determined from the two different approaches used to measure degradation rates.

The question of intracellular localization of this proteolytic process in mammalian cells has been a subject of intense interest and the results of various studies indicate that either degradation occurs in the ER or that both the ER and a post-ER compartment are degradation sites (see INTRODUCTION). Our studies with A1Pi-transformed Sec^{ts} mutants agree with the latter. The Sec18 gene encodes the yeast homologue to NSF (Eakle *et al.*, 1988), a factor required for the fusion of transport vesicles with their accepter membranes, and Sec17 appears to encode a second factor required for vesicle fusion (Griff *et al.*, 1992). Therefore, if the accumulation of A1PiZ seen in Sec17 and Sec18 cells at 37°C was a stabilizing effect of these mutations on A1PiZ, this would suggest that degradation occurs not only in the ER but also in a second site reached by vesicular transport. Although these data provide only preliminary support for this hypothesis, they are in agreement with the findings of Le *et al.* (1990,1992) that indicated that degradation of A1PiZ in mammalian cells took place in the ER and also occurred following export from the ER.

Together, the present data suggests that this process of selective degradation is not restricted to the ER but may be continuous as proteins traverse the exocytic pathway; different substrates could then be degraded according to their individual characteristics or different proteases could be specifically localized along the pathway. However, based on intracellular localization studies, substrates of this degradation pathway are known to accumulate in the ER. How then would these substrates proceed by vesicle transport to subsequent compartments for degradation? Alternatively, one cannot rule out the possibility that the accumulation of A1PiZ produced by blocking vesicle transport may result not from a block in delivery of substrate to a degradative compartment but through some secondary effect on proteolysis or other metabolic process.

Finally, although numerous biochemical and pharmacological studies have been carried out on various systems, a clear picture of the intracellular localization and molecular mechanisms of this selective proteolytic process has not emerged. It is clear though that identification of the relevant proteases will be extremely important for resolving the many questions relative to the elucidation of this degradation pathway. To this end, we have isolated mutant strains of yeast that show an ER protein degradation-deficient phenotype (McCracken and Karpichev, 1992). We predict that characterization of some of these mutants will reveal the proteases involved in this important intracellular degradative pathway.

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