Yeast Mutants Deficient in ER-Associated Degradation of the Z Variant of Alpha-1-Protease Inhibitor

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ABSTRACT

Saccharomyces cerevisiae mutants deficient in degradation of alpha-1-proteinase inhibitor Z (A1PiZ) have been isolated and genetically characterized. Wild-type yeast expressing A1PiZ synthesize an ER form of this protein that is rapidly degraded by an intracellular proteolytic process known as ER-associated protein degradation (ERAD). The mutant strains were identified after treatment with EMS using a colony blot immunoassay to detect colonies that accumulated high levels of A1PiZ. A total of 120,000 colonies were screened and 30 putative mutants were identified. The level of A1PiZ accumulation in these mutants, measured by ELISA, ranged from two to 11 times that of A1PiZ in the parent strain. Further studies demonstrated that the increased levels of A1PiZ in most of the mutant strains was not the result of defective secretion or elevated A1PiZ mRNA. Pulse chase experiments indicated that A1PiZ was stabilized in several strains, evidence that these mutants are defective in ER-associated protein degradation. Genetic analyses revealed that most of the mutations were recessive, ~30% of the mutants characterized conformed to simple Mendelian inheritance, and at least seven complementation groups were identified.

BECAUSE proteolysis plays an important role in the control of biological processes, it is important that we have a complete understanding of the mechanisms of the various cellular degradation pathways. Recently, a selective protein degradation process that operates in an early compartment of the secretory pathway has been described in mammalian cells (reviewed in KLAUSNER and SITIA 1990) and for yeast (FINGER *et al.* 1993; MCCRACKEN and KRUSE 1993; HAMPTON and RINE 1994). This process is a component of the quality control of newly synthesized proteins in the endoplasmic reticulum (ER). It is highly selective in that certain subunits of protein complexes synthesized in excess of the stochiometric requirement as well as aberrant proteins that accumulate in the ER are degraded by this ER-associated protein degradation process (ERAD), while most secretory proteins and ER residents are unaffected (reviewed in BONIFACINO and KLAUSNER 1994). Although the nature of this remarkable substrate selectivity is not yet known, there is evidence that the ER chaperone calnexin is involved, which indicates that molecular chaperones might play a role in ERAD (MCCRACKEN and BRODSKY 1996). Previous studies demonstrated that the proteolytic activity of ERAD was independent of the proteases of the mammalian lysosome and the yeast vacuole, suggesting that ERAD involved unidentified proteases within the ER. More recent evidence indicates that the cytosolic proteasome complex provides the proteolytic activity for ERAD in yeast (HILLER *et al.* 1996; WERNER *et al.* 1996) and in mammalian cells for some substrates (JENSEN *et al.* 1995; WARD *et al.* 1995; WIERTZ *et al.* 1996; for reviews see BRODSKY and MCCRACKEN 1997; MCCRACKEN *et al.* 1997).

Hence, the results from many studies have clearly established the existence of this selective proteolytic pathway, yet many questions regarding the molecular mechanisms remain unanswered. We chose a genetic approach to address this problem and report here the isolation and genetic characterization of yeast mutants deficient in the degradation of alpha 1-proteinase inhibitor Z (A1PiZ), a variant form of the human protein known to be a substrate for ERAD in mammalian cells and in yeast (LE *et al.* 1992; MCCRACKEN and KRUSE 1993).

MATERIALS AND METHODS

Materials and cell culture: The *Saccharomyces cerevisiae* strains used were: BC212 (*MATα ura3-52 leu2-3,112 his3-Δ 1 ade2-1*); BC159 (*MAT***a** *ura3-52 leu2-3,112 his3-Δ 1 ade2-1*); RSY269 (*MATα sec7-1 ura3-52 his4-619*), provided by R. SCHEKMAN, University of California, Berkeley; DBY1034 (*MAT***a** *ura3-52 his4-539 lys2-801*); and DBY2449 (*MATα ura3-52 ade2-101 suc2-9*), provided by D. PREUSS and D. BOTSTEIN, Stanford. Strains were cultured on synthetic medium supplemented

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with casein hydrolysate following the procedures of SHERMAN *et al.* 1986. Plasmids, pYES2.A1Pi, containing the cDNA sequence for A1PiM or A1PiZ under control of the *GAL1* promoter, were constructed previously (MCCRACKEN and KRUSE 1993). Cells were transformed according to the lithium acetate procedure (ITO *et al.* 1983) and were grown on medium containing 2% galactose to induce A1Pi expression. Cells lacking plasmid were obtained from transformed cells by growth in uracil containing medium. Mutagenesis was carried out by established techniques (LAWRENCE 1991). Briefly, cells were treated for 30 min with 30 μl/ml ethyl methane sulfonate to yield 45–60% survival (IK series), or 20 μl/ml ethyl methane sulfonate for 60–70% survival (JM series).

Colony-blot immunoassay for detection of A1Pi: The procedure was a modification from previous procedures (LYONS and NELSON 1984; MCCRACKEN and BROWN 1984). Cells grown on nitrocellulose discs were lysed *in situ* by 30-min incubation on 0.2 M NaOH, 0.1% SDS, and 0.05% 2-mercaptoethanol. The nitrocellulose disc was then washed and blocked in 50 mM tris pH 7.4, 150 mM NaCl, 0.2% Tween-20, and 0.2% Gelatin, prior to incubation with rabbit anti-human A1Pi (Dako, Carpenteria, CA). Discs were washed and incubated with horseradish peroxidase conjugated goat anti-rabbit immunoglobulin (United States Biochemical Co., Cleveland, OH), washed with 50 mM Tris, pH 7.4 and treated with a substrate solution (50 mM Tris pH 7.4, 0.6 mg/ml Diaminobenzidine, 0.03% $NiCl_2$, 0.1% H_2O_2). The intensity of the immuno-stain was scored relative to the control strains; wild-type cells expressing A1PiM = 4 (dark), wild-type cells expressing A1PiZ = 1 (light), and wild-type cells transformed with the pYES2.0 vector without A1Pi gene insert = 0.

Genetic analysis: Standard procedures and medium were used for construction and sporulation of diploids (SHERMAN *et al.* 1986), and spores were isolated using a micro-manipulator. Segregation of auxotrophic markers was followed by replica plating colonies onto selective medium and the mating type of each segregant was determined by crossing to mating type tester strains DC14 (*MAT***a** *his 1*) and DC17 (*MATα his 1*).

Enzyme-linked immunoadsorbent assay and cell lysate preparation: ELISA was performed in triplicate as previously described (MCCRACKEN *et al.* 1989). Cell lysates were prepared from 25 OD_{600} units of cells washed in 10 mM sodium azide, pelleted and resuspended in 40 mM sorbitol, 150 mM potassium acetate, 0.5 mM ethylene glycol-bis(β-aminoethyl ether) *N,N,N,N*-tetraacetic acid (EGTA), 20 mM HEPES, 2 mM magnesium chloride, pH 7.5, 0.5 M phenylmethylsulfonyl fluoride (PMSF), 0.1 mM N-tosyl-l-phenylalanine chloromethyl keytone (TPCK), 1 mM benzamidine HCL, 25 μM pepstatin A, and 1 mg/ml lyticase at 37° for 2 hr. Triton X-100 was added to a final concentration of 1% and samples were incubated on ice for a minimum of 1 hr. Lysates were microcentrifuged at 10,000 × *g* for 5 min at 4° and the supernatant was collected and diluted 1:1 with protease inhibitor cocktail [32 mM HEPES pH 7.6, 10 mM ethylenediaminetetraacetic acid (EDTA) pH 7.6, 0.5 mM PMSF, 0.1 mM TPCK, 1 mM benzamidine HCl, and 25 μM pepstatin A].

Pulse chase protein radiolabeling and Northern analyses: Cells were labeled with 15-20 μCi/ml ^{35}S-methionine and lysates prepared as above were immunoadsorbed with A1Pi-specific antibody as previously described (MCCRACKEN and KRUSE 1993). Immuno-reactive proteins were resolved by SDS-PAGE and visualized by either autoradiography and densitometry or phosphor imaging using a BioRad PhosphorImager (BioRad, Hercules, CA). First order decay curves, generated using Cricket Graph software (Cricket Software, Malvern, PA) were used to determine the half-life of A1PiZ. Whole cell RNA, purified from cells cultured in galactose containing medium for 7 hr to induce the expression of A1PiZ, was analyzed by Northern blotting following standard procedures (SAMBROOK et al. 1989). Each blot was probed first with ^{32}P-labeled *A1Pi* cDNA, exposed to film, and then washed and probed a second time with ^{32}P-labeled *TRP1* cDNA. Following hybridization to the ^{32}P-labeled *TRP1* cDNA, the blot was exposed to film longer than when hybridized to the A1PiZ probe. The autoradiograms were analyzed by densitometry using a Hoeffer GS300 scanning densitometer (San Francisco, CA).

Invertase secretion analyses: Yeast strains were grown in synthetic medium supplemented with casein hydrolysate and 2% lactic acid (pH 5.5) at 25, 30, or 37°. Cell walls were removed as described by PRINGLE *et al.* (1991) and spheroplasted cells were separated from their periplasmic fraction by centrifugation. Spheroplasts were suspended in 0.1 M phosphate buffer (pH 7.2) and lysed by three times freeze/thaw at −70°. Spheroplasts and periplasmic fractions were assayed for invertase activity based on the method described by GOLDSTEIN and LAMPON (1975). To 1.6 ml of each fraction, 100 μl of 0.5 M sucrose was added, and each fraction was allowed to incubate for 30 min at 37° with gentle shaking. Sucrose hydrolysis was terminated by boiling the samples for 10 min. Once cooled, liberated glucose was quantified by incubation for 45 min at room temperature with 1 ml of 10 mM potassium phosphate buffer (pH 7.2) containing 300 ng o-dianisidine, 50 ng glucose oxidase, and 10 ng horseradish peroxidase. The colorimetric reaction was terminated by addition of 1.0 ml of 6 N HCl, and absorbance was measured at 450 nm.

Nomenclature: The following gene and allele numbers have been assigned to the A1PiZ degradation-deficient strains: JM8 carries *add1-1*; JM30, *add2-1*; JM54, *add3-1*; JM62, *add4-1*; JM103, *add5-1*; JM134, *add6-1*; IK 10, *add7-1*; and IK13/3a, *add3-2*.

RESULTS

Isolation of mutants with increased accumulation of A1PiZ: Wild-type strains BC212 and DBY1034 expressing A1PiZ from the plasmid pYES2.A1PiZ were exposed to EMS, and surviving colonies were screened using a colony-blot immunoassay that allowed detection of accumulated A1PiZ (MATERIALS AND METHODS). Thirty putative mutant clones that showed a more intense immuno-stain were identified from two screens of ~60,000 colonies each. The accumulation of A1PiZ in the mutant strains, measured by ELISA, was two- to 11-fold greater than that of nonmutagenized wild-type cells (Table 1), demonstrating that the colony-blot immunoassay was an accurate screening method to detect colonies with elevated levels of A1PiZ.

To determine whether the mutations responsible for A1PiZ accumulation were in the *A1PiZ* expression vector or in the genome, mutant strains were cured of the initial *A1PiZ* expression vector and retransformed with a vector that had not been exposed to mutagen. The retransformed strains displayed elevated levels of A1PiZ that were virtually identical to the original mutant isolates (Table 1), indicating that the mutations responsible for A1PiZ accumulation were genomic.

To ensure that increased accumulation of A1PiZ was not due to increased transcription of the gene, we examined the levels of *A1Pi*-specific RNA in these mutants by Northern blot analyses. As an internal control, the

TABLE 1

Levels of A1PiZ and secretion of invertase

	A1PiZ ELISA[a]			Invertase activity[b]			
				μg/OD		Ratio I/E	
Mutant	ng/OD	Xwt	RT#[c]	I	E	25°	37°
IK wt	1.11		1.08	22 ± 2	66 ± 2	0.33	0.42
IK3	2.75	2.5	2.04	44 ± 1	51 ± 3	0.88	0.62
IK4	4.94	4.5	2.84	47 ± 2	152 ± 9	0.31	0.28
IK5	4.33	3.9	4.93	14 ± 2	12 ± 2	1.19	0.80
IK9	3.90	3.5	ND	29 ± 5	317 ± 3	0.09	0.10
IK10	12.50	11.3	11.78	3 ± 2	65 ± 1	0.05	0.06
IK11	4.35	3.9	3.77	22 ± 2	71 ± 5	0.31	0.21
IK12	2.65	2.4	3.03	10 ± 1	13 ± 2	0.79	0.56
IK13	7.08	6.4	6.25	32 ± 2	73 ± 4	0.44	0.45
IK15	2.46	2.2	2.15	19 ± 2	35 ± 3	0.55	0.43
IK16	8.62	7.8	8.70	46 ± 1	51 ± 1	0.88	0.69
IK17s	3.33	3.0	2.62	20 ± 1	27 ± 6	0.73	0.55
IK17w	4.50	4.1	4.22	22 ± 3	41 ± 1	0.53	0.55
IK18	3.66	3.3	3.42	20 ± 1	53 ± 1	0.39	0.36
IK19	5.96	5.4	5.93	24 ± 1	33 ± 1	0.72	0.47
IK23	3.66	3.2	2.54	17 ± 1	18 ± 2	0.95	0.69
IK24	3.67	3.2	3.61	40 ± 2	68 ± 8	0.60	0.45
IK27	10.32	9.3	ND	52 ± 1	57 ± 4	0.91	0.44
JM wt	5.15		4.98	26 ± 3	62 ± 4	0.42	0.49
JM8	10.20	2.0	11.21	24 ± 1	64 ± 5	0.36	0.32
JM54	12.95	2.5	10.34	32 ± 7	270 ± 6	0.14	0.12
Sec7				26 ± 2	31 ± 1	0.84	1.99

Parent strains of the IK mutants (IK wt), and of the JM mutants (JM wt) are BC212 and DBY1034 strains, respectively, transformed with pYES2.A1PiZ. I/E indicates internal (speroplast) to external (periplasm) ratios and are presented for experiments performed at both 25 and 37°; ND, experiments were not performed. OD values are means ± SD.

[a] A1PiZ was quantified by ELISA as described in MATERIALS AND METHODS, values are ng A1PiZ per OD_{600} units of cells.

[b] Invertase activity measured is micrograms of glucose production per OD_{600} unit of cells examined at 25°.

[c] The amount of A1PiZ in re-transformed strains.

relative level of *TRP1* mRNA was determined on the blot and a ratio of *A1Pi* mRNA to *TRP1* mRNA was calculated and averaged from two experiments. This ratio in most mutant strains (0.45–0.64) was similar to the 0.50 ratio observed in wild-type cells, except for mutant IK15 in which the ratio was 1.05. Thus, transcriptional changes were unlikely as the explanation for enhanced A1PiZ accumulation in most mutants.

To determine whether the secretory pathway was intact, we examined the secretion of invertase (Table 1). As a control, the ratio of internal invertase activity (I) to secreted invertase activity (external, E) was determined for a secretion defective *sec7* strain (FRANZUSOFF and SCHEKMAN 1989). At the permissive temperature 25°, *sec7* cells express a partial defect in secretion that is demonstrated in these experiments by the high ratio of internal to secreted invertase activity and that is exacerbated at the nonpermissive temperature, 37° (IE in Table 1). Mutants IK3, 5,16, 23, and 27 had an internal to external invertase ratio equal to or greater than that of *sec7* cells, suggesting that they had defective secretory functions and were thus excluded from further study for this project. Interestingly, three mutants (IK9, IK10, and JM54) appeared to be more efficient secretors of invertase than wild-type cells. The results of the secretion analyses demonstrated that the increased accumulation of A1PiZ by many mutants was not due to inhibition of protein transport in the secretory pathway.

The degradation of A1PiZ was examined in wild-type and selected mutant strains by pulse-chase protein radiolabeling (Figure 1A). The decay curves generated from the pulse-chase analyses and the half-lives of A1PiZ calculated from these data (Figure 1B) demonstrated that A1PiZ was not stabilized in mutants IK4 and JM72, indicating that these strains were not defective in degradation but accumulated elevated levels of A1PiZ for reasons not examined in this study. However, we found that A1PiZ was stabilized two- to threefold in mutants IK10, IK13, IK17w, JM8, and JM54, demonstrating that these mutants are deficient in the degradation of A1PiZ.

To test whether the mutations would lead to stabilization of other ERAD substrates, cured strains of mutants

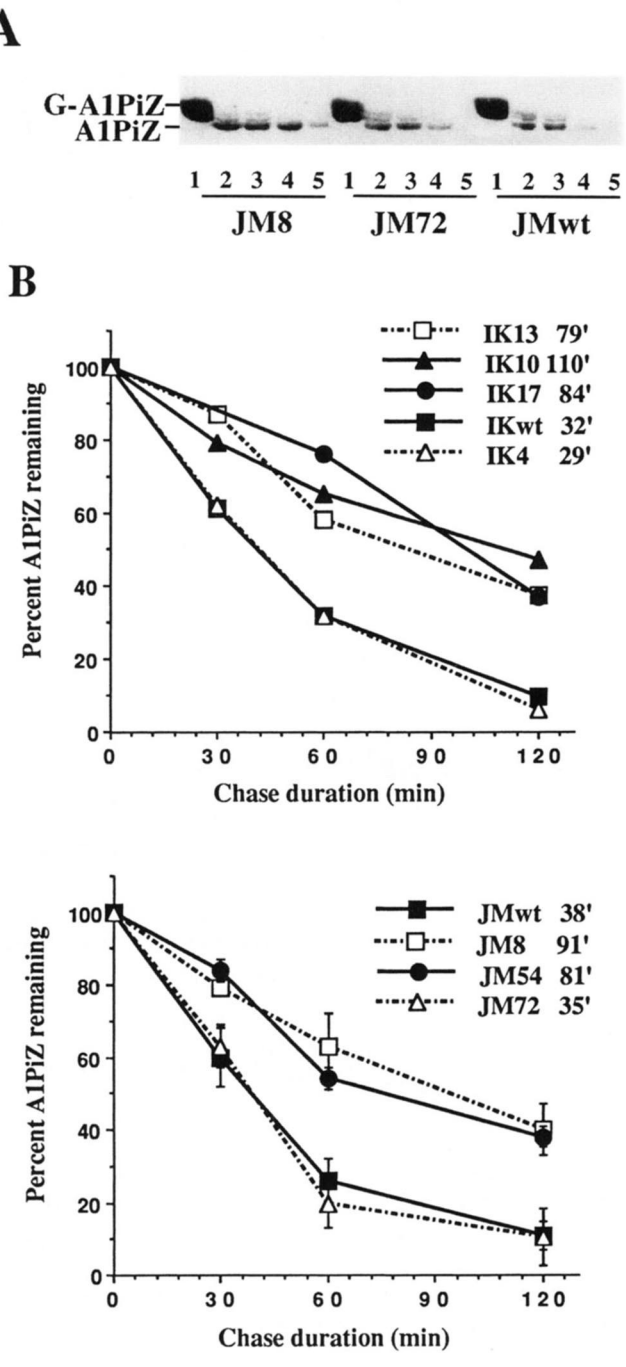

FIGURE 1.—Pulse-chase protein radiolabeling of A1PiZ in mutant and wild-type cells. (A) The autoradiogram shows immunoprecipitated radiolabeled A1PiZ produced in mutants JM8, JM72, and JMwt strains expressing A1PiZ. Cells labeled for 45 min, were chased for zero time (lanes 1 and 2), 15 min (lanes 3), 30 min (lanes 4), or 60 min (lanes 5). All samples, except those in lanes 1, were treated with endoglycosidase-H to remove the three carbohydrate chains added to A1PiZ in the ER. G-A1PiZ indicates glycosylated and A1PiZ indicated deglycosylated A1PiZ. Lanes 1 were loaded with excess protein in order to visualize a possible heterogeneous population of high molecular weight Golgi-processed forms of A1PiZ (MOIR and DUMAIS 1987). No high molecular weight A1PiZ was detected and this portion of the gel has been removed. (B) Decay curves for A1PiZ in several JM and IK series mutants. Strains were examined by pulse-chase protein radiolabeling as shown in A. Density values of bands in lanes containing endo-H-treated samples were averaged from two or more experiments and are presented with standard error bars. Half-lives (in minutes) generated from these data are presented to the right of each strain identification on the figure legend.

TABLE 2

Stabilization of ERAD substrates

Mutant	A1PiZ (ng/OD)	A1PiM (ng/OD)	Inv 538 (μg/OD)
IK10	12.50	122.90	9.53
IK13	7.08	76.99	7.30
IK17w	4.50	62.86	5.94
IK wt	1.11	47.00	3.53

Numbers represent amounts of protein as measured by ELISA (see MATERIALS AND METHODS). Results are averages of triplicate assays performed at last three times. The SD was typically <25% of the mean.

IK10, IK13 and IK17w were transformed with genes encoding proteins known to be ERAD substrates; A1PiM (MCCRACKEN and KRUSE 1993) and Suc2-538, a truncated yeast invertase that is inefficiently secreted and accumulates in the ER (PREUSS *et al.* 1991). The intracellular accumulation of these proteins was monitored by ELISA. We found that both of these proteins were stabilized in each of these mutants (Table 2), suggesting a general defect in ERAD.

Genetic characterization of A1PiZ degradation-deficient mutants: The immunoassay was used to determine whether the mutations were dominant or recessive and whether single gene mutations were responsible for the A1PiZ accumulation phenotypes. The results are presented for JM8 in Figure 2. The immuno-stain for the mutant is dark and for the wild-type and heterozygous diploid, it is faint, indicating that the mutation in JM8 is recessive. Similar analyses were performed with other mutants (Tables 3 and 4).

To examine the number of genes involved in the mutant phenotype, mutants were crossed to an isogenic wild-type strain, the resulting diploids sporulated, and tetrads dissected and scored for A1PiZ levels by immunoassay. Of 14 tetrads for the cross of JM8 to an isogenic wild type, 13 segregated 2:2, indicating the presence of a single mutation in JM8 causing the A1PiZ accumulation phenotype. Similar tetrad analysis of each JM mutant indicated that many mutants (8, 30, 54, 72, 134) displayed phenotypes likely to be controlled by single mutations (Table 3). Some of the aberrant tetrads were probably a result of scoring error due to limitations of the immunoassay.

A surprising but intriguing result was obtained when tetrad dissection was performed with the IK series of mutants. Many, if not all, of these mutants did not display the 2:2 phenotypic segregation pattern expected for single gene mutations. Indeed, most expressed a high per-

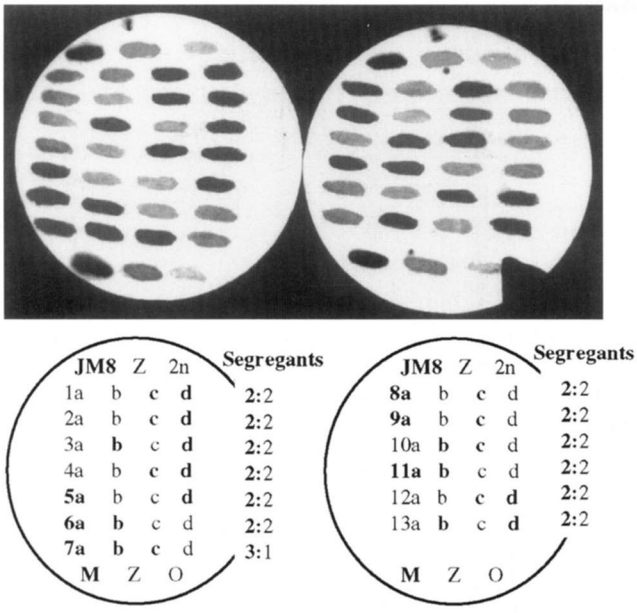

FIGURE 2.—Tetrad analysis of JM8 × wild type examined by colony-blot immunoassay. Spores (a–d) from dissected tetrads (indicated as 1–13) streaked and grown on nitrocellulose discs were processed for immunoblot analyses as described in MATERIALS AND METHODS. Control strains at top from left are: mutant (JM8), wild-type strain expressing A1PiZ (Z), and the diploid JM8 × JMwt, (2n). Control strains, at bottom, are wild-type strains expressing A1PiM (M), or A1PiZ (Z), or transformed with the pYES2.0 vector without an A1Pi gene insert.

centage of aberrant segregation patterns, suggesting that mutations in two or more genes were responsible for the A1PiZ accumulation phenotype. Thus, to determine if two genes could be involved, the immunoblots were evaluated considering an intermediate phenotype, *i.e.*, a stain intensity between that of the wild type and mutant. Using this model, the corresponding genotypes would be: wild type (*AB*), intermediate (*Ab* or *aB*), and mutant (*ab*). Three classes of tetrads would be produced by a diploid that is heterozygous for two markers: parental ditype (PD) 2 *AB : 2 ab*; nonparental ditype (NPD) 2 *Ab:2 aB*, in which all spore clones display an intermediate phenotype; and tetratype (T) 1 AB:1 Ab:1 aB:1 ab, in which there is one spore clone that stains lightly, two with intermediate staining, and one that stains darkly. Results of these analyses suggested that many of the IK mutants displayed a phenotype that appeared to be controlled by two genes (Table 4).

To test the two gene hypothesis more closely, the phenotypic segregation pattern was examined in tetrads from diploids made by crossing IK13 segregants from putative NPD tetrads to each other. The presence of gene *a* or gene *b* in putative singly mutant spores was determined by examining the segregation patterns of dissected tetrads. For example, if gene *a* was present in both segregants, the expected genotype of all spores produced from this cross would be aB and they would all show an intermediate intensity stain. If gene *a* was present in one segregant and gene *b* was present in the other, the resulting tetrads should show a P:N:T ratio of 1:1:4, assuming an absence of linkage or centromere linkage (for at least one). The results shown in Table 5 suggest that two independently segregating genes controlled the A1PiZ accumulation phenotype of IK13. However, analyses of the segregant crosses was complicated by the limitations of the immunoassay when working with low-intensity signals and by the small number of tetrads analyzed. Nevertheless, the simplest interpretation of the results is that IK13/3b and IK13/3d were

TABLE 3

Genetic analyses of the JM series of mutants

Mutant	No. of tet	Percent V	Signal	Allele	Tetrad types[a]				
					4:0	3:1	2:2	1:3	0:4
JM8	14	88	4	rec	0	0	13	1	0
JM54	12	43	4	rec	0	0	10	2	0
JM72	18	75	4	rec	1	1	14	2	0
JM72w	9	45	4	rec	0	1	8	0	0
JM16	19	68	3	rec	2	0	14	3	0
JM30	12	46	3	rec	1	0	9	2	0
JM36	6	10	3	dom	0	2	4	2	0
JM134	10	28	3	rec	0	2	8	0	0
JM62	13	54	3	rec	1	4	7	1	0
JM103	16	57	3	rec	3	7	6	0	0
JM60	13	57	2	rec	1	4	6	2	0
JM90	22	79	2	rec	0	7	13	2	0
JM9	17	61	2	rec	1	2	12	0	2

Mutants were crossed to a wild-type strain, the resulting diploids sporulated, and tetrads dissected and scored for A1PiZ levels by immunoassay. No. of tet, number of tetrads analyzed by colony-blot immunoassay; percent V, percent of dissected tetrads with four viable spores; Signal, intensity of the immuno-stain, wild type = 1; Allele, dominant or recessive mutation.

[a]Number of tetrads with indicated number of wild-type to mutant spores.

TABLE 4

Genetic analyses of the IK series of mutants

Mutant	No. of tet	Percent V	Signal	Allele	Tetrad types[a] PD 2:0:2	NPD 0:4:0	T 1:2:1	4:0:0
IK3	14	54	2	rec	3	6	5	
IK4	10	57	2	rec	2	3	5	
IK5	10	65	3	rec	4	1	5	
IK9	12	45	2	rec	2	5	5	
IK10	16	52	3	rec	3	3	10	
IK11	9	68	2	rec	2	1	6	
IK12	14	54	3	rec	6	2	6	
IK13	10	71	4	rec	2	2	6	
IK15	9	36	2	rec	2	2	5	
IK16	19	95	2	rec	5	5	9	
IK17s	10	77	3	rec	3	1	6	
IK17w	12	71	3	rec	3	1	8	
IK18	9	50	2	dom	2	3	4	
IK19	12	54	2	rec	2	2	8	
IK23	19	79	2	dom	6	3	10	
IK24	17	77	3	rec	4	4	8	1
IK27	15	58	2	rec	4	2	9	

Mutants were crossed to a wild-type strain, the resulting diploids sporulated, and tetrads dissected and scored for A1PiZ levels by immunoassay. No. of tet, number of tetrads analyzed by colony-blot immunoassay; percent V, percent of dissected tetrads with four viable spores; signal, intensity of the immuno-stain, wild type = 1; allele, dominant or recessive mutations.

[a] Number of tetrads with indicated number of wild-type to intermediate to mutant spores; PD, parental ditype; NPD, nonparental ditype; and T, tetratype.

carrying the same mutant allele while IK13/3a and IK13/3c carried the other.

To further test this hypothesis, spore clones from the putative NPD tetrads were backcrossed to the parent strain, IK13, and to a wild-type strain. A segregant (aB or Ab) from a NPD tetrad, when crossed to IK13 (ab), will make a diploid that should produce tetrads with two intermediate staining, two dark staining (mutant), and no wild-type (light staining) spore clones. These same segregants when crossed to wild type should give diploids that produce two wild-type and two intermediate staining spore clones. The results shown in Table 5, largely bear out these expectations. Taken together, these findings are strong evidence that mutant IK13

TABLE 5

Genetic characterization of mutant IK13

Crosses	No. of tet	Tetrad types[a] 2:0:2	0:4:0	1:2:1	Aberrant segregations
Segregant × Segregant		2:0:2	0:4:0	1:2:1	
IK13/3a × 1K13/3b	19	3	3	13	
IK13/3b × IK13/3d	10	1	7	2	
IK13/3c × IK13/3d	15	1	4	8	2
IK13/3a × IK13/3d	6	1		5	
Segregants × Mutant IK13		0:2:2			
IK13/3a × IK13/9c[b]	12		9		3
IK13/3b × IK13/7c	14		12		2
IK13/3c × IK13/7c	12		12		
IK13/3d × IK13/9c	11		8		3
Segregant × wild type			2:2:0		
IK13/3b × DBY2449	14		9		5
IK13/3c × DBY2449	15		11		4

IK13 segregants from putative NPD tetrads were crossed to each other (Segregant × Segregant), to mutant IK13 (Segregant × Mutant IK13), and to a wild-type strain (Segregant × Wild type), the resulting diploids sporulated, and the tetrads were dissected and scored for A1PiZ levels by immunoassay. No. of tet, the number of tetrads analyzed by colony-blot immunoassay.

TABLE 6

Complementation analyses

Mutant	JM8 (*add1-1*)	JM30 (*add2-1*)	JM54 (*add3-1*)	JM62 (*add4-1*)	JM103 (*add5-1*)	JM134 (*add6-1*)	IK10 (*add7-1*)	IK13/3a (*add3-2*)
JM8	−	+	+	+	+	+	+	+
JM30	+	−	+	+	+	+	+	+
JM54	+	+	−	+	+	+	+	−
JM62	+	+	+	−	+	+	+	+
JM103	+	+	+	+	−	+	+	+
JM134	+	+	+	+	+	−	+	+
IK10	+	+	+	+	+	+	−	+
IK13/3a	+	+	−	+	+	+	+	−

+, complementation, *i.e.*, suggests different mutated genes; −, no complementation, *i.e.*, suggests same mutated gene.

carries two independently segregating genes, each of which results in A1PiZ accumulation. Based on this analysis of IK13 and the tetrad analyses of the other IK mutants, we believe that the A1PiZ accumulation phenotype of other IK mutants may also be the result of mutations in two genes.

To begin to address the number of different genes that may be involved in the A1PiZ accumulation phenotype, certain mutants were subjected to complementation analysis. Outcrossed mutants with appropriate genetic markers and mating type were selected and crossed to each other until all possible combinations were obtained in diploid cells. The phenotypes of the resulting diploids were then analyzed by colony-blot immunoassay to test for complementation. Results of the immunoassay indicated that complementation occurred in all but one combination, JM54 × IK13/3a (Table 6). The other strains tested by complementation analysis had mutant genes that were not allelic to each other, indicating seven different complementation groups.

DISCUSSION

We have isolated and characterized *S. cerevisiae* mutant strains that are defective in the degradation of A1PiZ and propose to name the genes involved *ADD* for A1PiZ degradation-deficient.

The colony-blot immunoassay screening protocol devised for these studies provided an effective method to identify *add* mutants and was also useful for genetic characterization. Both the steady state levels and the half-lives of A1PiZ were increased in the *add* mutants *vs.* wild type. However, the steady state accumulation of A1PiZ was greater than would be predicted by the determined half-lives. It is likely that this difference is due to the aggregation of A1PiZ when expression levels exceed the amount that can be processed by the ER quality control. A1PiZ is known to aggregate into a nondegradable species that accumulates in inclusion bodies of liver cells (BATHURST *et al.* 1984; CALLEA *et al.* 1984).

Genetic analyses suggested that for many mutants the *add* phenotype was due to recessive single gene mutations, while others appeared to have mutations in two genes, each of which conferred a lesser defect on its own. Complementation analyses between the strains with recessive mutations indicated that eight strains carried mutations in seven different complementation groups. This result was not surprising since large numbers of genes have been identified in other secretory pathway processes. For example, in a screen for temperature-sensitive secretion-defective yeast mutants, NOVICK and colleagues (1980) found 23 complementation groups; yet today, >60 genes have been identified that are involved in the secretory pathway.

In conclusion, we have presented compelling evidence that the accumulation of A1PiZ in the add mutants reflects a defect in the ER-associated protein degradation process. Thus, the isolation of these *add* mutants provides the genetic ground work for understanding the molecular mechanism of this process. In addition, the identification of at least seven complementation groups among eight mutants indicates that saturation was not reached, making it likely that many more genes may be involved. Thus, this first glimpse at the complexity of the ER-associated protein degradation pathway suggests that many gene products may be required.

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LITERATURE CITED

BATHURST, I. C., J. TRAVIS, P. M. GEORGE and R. CARRELL, 1984 Structural and functional characterization of the abnormal Z alpha-1-antitrypsin isolated from human liver. FEBS Lett. **177:** 179–183.

BRODSKY, J. L., and A. A. MCCRACKEN, 1997 ER-associated and proteasome-mediated protein degradation: how two topologically restricted events came together. Trends Cell Biol. (in press).

BONIFACINO, J. S., and R. D. KLAUSNER, 1994 Degradation of pro-

teins retained in the endoplasmic reticulum, pp. 137–169 in, *Modern Cell Biology, Cellular Proteolytic Systems*, Vol. 15, edited by A. CIECHANOVER and A. L. SCHWARTZ. Wiley-Liss Inc. New York.

CALLEA, F., J. FEVERY, G. MASSI, C. LIEVENS, J. DE GROOTE *et al.*, 1984 Alpha-1-antitrypsin (ATT) and its stimulation in the liver of PiMZ phenotype individuals. A "recruitment-secretory block" phenomenon. Liver **4:** 325–337.

FINGER, A., M. KNOPP and D. H. WOLF, 1993 Analysis of two mutated vacuolarproteins reveals a degradation pathway in the ER or an ER-related compartment of yeast. Eur. J. Biochem. **218:** 565–574.

FRANZUSOFF, A., and R. SCHEKMAN, 1989 Functional compartments of the yeast Golgi apparatus are defined by the Sec7 mutation. EMBO J. **8:** 2695–2702.

GOLDSTEIN, A., and J. O. LAMPON, 1975 b-D-fructofuranoside fructohydrolase from yeast. Methods Enzymol. **42:** 504–511.

HAMPTON, R. Y., and J. RINE, 1994 Regulated degradation of HMG-CoA reductase, an integral membrane protein of the endoplasmic reticulum, in yeast. J. Cell Biol. **125:** 299–312.

HILLER, M. M., A. FINGER, M. SCHWEIGER and D. H. WOLF, 1996 ER-degradation of a misfolded luminal protein occurs via the cytosolic ubiquitin-proteasome pathway. Science (in press).

ITO, H., Y. FUKUDA, K. MURATA and A. KIMURA, 1983 Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153:** 163–168.

JENSEN, T. J., M. A. LOO, S. PIND, D. B. WILLIAMS, A. L. GOLDBERG *et al.*, 1995 Multiple proteolytic systems, including the proteasome, contribute to CFTR processing. Cell **83:** 129–135.

KLAUSNER, R. D., and R. SITIA, 1990 Protein degradation in the endoplasmic reticulum. Cell **62:** 611–614.

LAWRENCE, C. W., 1991 Classical mutagenesis techniques. Methods Enzymol. **194:** 273–281.

LE, A., G. A. FERRELL, D. S. DISHON, Q. Q. LE and R. N. SIFERS, 1992 Soluble aggregates of the human PiZ alpha 1-antitrypsin variant are degraded within the endoplasmic reticulum by a mechanism sensitive to inhibitors of protein synthesis. J. Biol. Chem. **267:** 1072–1080.

LYONS, S., and N. NELSON, 1984 An immunological method for detecting gene expression in yeast colonies. Proc. Natl. Acad. Sci. USA **81:** 7426–7430.

MCCRACKEN, A. A., and J. L. BRODSKY, 1996 Assembly of ER-associated protein degradation *in vitro*: dependence on cytosol, calnexin, and ATP hydrolysis. J. Cell Biol. **132:** 291–298.

MCCRACKEN, A. A., and J. L. BROWN, 1984 A filter immunoassay for detection of protein secreting cell colonies. BioTechniques **2:** 82–87.

MCCRACKEN, A. A., and K. B. KRUSE, 1993 Selective protein degradation in the yeast exocytic pathway. Mol. Biol. Cell **4:** 729–736.

MCCRACKEN, A. A., K. B. KRUSE and J. L. BROWN, 1989 Studies on the molecular basis for the defective secretion of the Z variant of human alpha-1-proteinase inhibitor. Mol. Cell. Biol. **9:** 1406–1414.

MCCRACKEN, A. A., E. D. WERNER and J. L. BRODSKY, 1997 ER-associated protein degradation, in *Advances in Molecular Cell Biology, Intracellular Protein Degradation*, edited by A. J. RIVETT. JAI Press Inc., Greenwich, CT. (in press)

MOIR, D. T., and D. R. DUMAIS, 1987 Glycosylation and secretion of human alpha-1-antitrypsin by yeast. Gene **56:** 209–217.

NOVICK, P., C. FIELD and R. SCHEKMAN, 1980 Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell **21:** 205–215.

PREUSS, D, J. MULHOLL and, C. A. KAISER, P. ORLEAN, C. ALBRIGHT, *et al.*, 1991 Structure of the yeast endoplasmic reticulum: localization of ER proteins using immunofluorescence and immunoelectron microscopy. Yeast **7:** 891–911.

PRINGLE, J. R., A. E. M. ADAMS, D. G. DRUBIN and B. K. HAARER, 1991 Immunofluorescence methods for yeast. Methods Enzymol. **194:** 565–601.

SAMBROOK, J., E. F. FRITCH and T. MANIATIS, 1989 *Molecular Cloning, A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

SHERMAN, F., G. F. FINK and J. B. HICKS, 1986 *Laboratory Course Manual for Methods in Yeast Genetics.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

WARD, C. L., S. OMURA and R. R. KOPITO, 1995 Degradation of CFTR by the ubiquitin-proteasome pathway. Cell **83:** 121–127.

WERNER, E. D., J. L. BRODSKY and A. A. MCCRACKEN, 1996 Proteasome-dependent ER-associated protein degradation: an unconventional route to a familiar fate. Proc. Natl. Acad. Sci. USA **93:** 13797–13801.

WIERTZ, E. J. H., T. R. JONES, L. SUN, M. BOGYO, H. J. GEUZE *et al.*, 1996 The human cytomegalovirus US11 gene product dislocates MHC Class I heavy chains from the endoplasmic reticulum to the cytosol. Cell **84:** 769–779.

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