Differential requirements of novel A1PiZ degradation deficient (*ADD*) genes in ER-associated protein degradation

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Summary

In the eukaryotic cell, a protein quality control process termed endoplasmic reticulum-associated degradation (ERAD) rids the ER of aberrant proteins and unassembled components of protein complexes that fail to reach a transport-competent state. To identify novel genes required for ERAD, we devised a rapid immunoassay to screen yeast lacking uncharacterized open reading frames that were known targets of the unfolded protein response (UPR), a cellular response that is induced when aberrant proteins accumulate in the ER. Six genes required for the efficient degradation of the Z variant of the α1-proteinase inhibitor (A1PiZ), a known substrate for ERAD, were identified, and analysis of other ERAD substrates in the six A1PiZ-degradation-deficient (*add*) mutants suggested diverse requirements for the Add proteins in ERAD. Finally, we report on bioinformatic analyses of the new Add proteins, which will lead to testable models to elucidate their activities.

Key words: ERAD, Protein quality control, Degradation

Introduction

Proteins destined for cellular export and integral membrane proteins enter the secretory pathway at the endoplasmic reticulum (ER). Once translocated across or into the ER membrane, the nascent polypeptide folds and may become disulfide bonded, modified with carbohydrate, proteolytically processed or assembled into a multimeric complex. ER-resident chaperones assist with both folding and modification, and they ensure that only correctly folded and completely assembled proteins progress to the Golgi apparatus en route to their final destinations (for reviews, see Hegde and Lingappa, 1999; Benham and Braakman, 2000; Ellgaard and Helenius, 2001). Aberrant proteins that fail to reach a transport-competent state are removed from the secretory pathway by ER-associated protein degradation (ERAD), whereby the protein substrate is exported, or 'retro-translocated', from the ER to the cytosol and degraded by the 26S proteasome (for reviews, see Brodsky and McCracken, 1999; Plemper and Wolf, 1999; Römisch, 1999; Hampton, 2000; Fewell et al., 2001).

Genetic screens for ERAD-defective *Saccharomyces cerevisiae* mutants (Hampton et al., 1996; Knop et al., 1996; McCracken et al., 1996), the use of mammalian cell-free systems (Winitz et al., 1996; Wilson et al., 2000; Gusarova et al., 2001; Shamu et al., 1999; Shamu et al., 2001; Ye et al., 2001) and a yeast in vitro ERAD assay (McCracken and Brodsky, 1996; Werner et al., 1996; Pilon et al., 1997; Gillece et al., 2000) have facilitated the discovery of components required for ERAD substrate specificity, protein export and delivery of substrates to the proteasome; however, unanswered questions regarding the molecular details of ERAD remain. Results from several laboratories reveal that both ubiquitination and molecular chaperones aid in the targeting of ERAD substrates to the proteasome, yet it appears that each substrate has a unique set of requirements for its degradation (reviewed by Fewell et al., 2001). For example, not all ERAD substrates are ubiquitinated (Werner et al., 1996). Furthermore, integral ER membrane proteins appear to be degraded independently of the ER molecular chaperone BiP (IgG heavy chain binding protein), whereas soluble ERAD substrates require BiP to mediate their export to the cytoplasm (Brodsky et al., 1999; Nishikawa et al., 2001). By contrast, the cytoplasmic heat-shock protein Hsp70 chaperone, Ssa1p, facilitates the degradation of several integral membrane proteins but is dispensable for the proteolysis of soluble substrates (Hill and Cooper, 2000; Zhang et al., 2001). Finally, genes required for the degradation of one substrate may or may not be required for the degradation of a related substrate (e.g. Wilhovsky et al., 2000). Clearly, a greater number of substrates and factors required for ERAD must be analyzed to better understand the molecular mechanisms of this pathway. By analogy, the full spectrum of factors required for protein transport and a more complete understanding of the secretory pathway emerged only after varied genetic screening protocols and biochemical attacks were employed to examine the transport of multiple diverse cargoes (e.g. Schekman and Orci, 1996).

One soluble ERAD substrate is an unglycosylated version

of the yeast mating pre-pheromone, pre-pro alpha factor (ppαF). After signal sequence cleavage, ppαF is converted to pro-alpha factor (pαF) and if glycosylation is prevented, pαF is retro-translocated to the cytoplasm and destroyed by the proteasome (Werner et al., 1996). An assay in which the degradation of pαF was faithfully reconstituted indicated requirements for the ER-resident chaperones calnexin (McCracken and Brodsky, 1996), protein disulfide isomerase (PDI) (Gillece et al., 1999) and BiP (Brodsky et al., 1999). Ssa1p was dispensable for pαF degradation, as was polyubiquitination (Werner et al., 1996; Brodsky et al., 1999). Indeed, the 19S cap of the proteasome is sufficient for ATP-mediated pαF retro-translocation, and the substrate can be degraded on addition of the 20S particle (R. Lee and J.L.B., unpublished).

The mutant Z variant of α1-proteinase inhibitor (A1PiZ, also known as α1-antitrypsin-Z or AT-Z) is another soluble substrate for ERAD (McCracken and Kruse, 1993; McCracken et al., 1996; Teckman and Perlmutter, 1996). In humans, secretion-incompetent A1Pi mutants may aggregate in the hepatic ER, ultimately giving rise to liver disease and juvenile emphysema, although it is unclear how the misfolded protein is converted from being an ERAD substrate into an aggregation-prone polypeptide (Lomas et al., 1992; Yu et al., 1995).

To begin to dissect A1Pi maturation at the molecular level, both A1PiZ-expressing human cell lines and yeast have been used. In yeast, optimal A1PiZ degradation requires the ER-resident molecular chaperone BiP, yet is degraded in the absence of functional Ssa1p (Brodsky et al., 1999). Ubiquitination does not appear to be required for A1Pi degradation in yeast, whereas in mammalian cells both ubiquitin-dependent and ubiquitin-independent A1PiZ degradation have been described (Teckman et al., 2000). Oligosaccharyl trimming and the ER resident chaperone calnexin also play a role in the ERAD of A1PiZ (Qu et al., 1996; Marcus and Perlmutter, 2000; Cabral et al., 2000).

The proteolysis of a third soluble ERAD substrate, a mutated form of carboxypeptidase Y (CPY*), also requires BiP in yeast (Plemper et al., 1997). Additional factors involved in CPY* degradation include an integral ER membrane protein of unknown function, Der1p (Knop et al., 1996), two proteins involved in transport between the ER and Golgi, Erv29p and Erv14p (Caldwell et al., 2001), and Cdc48p, a cytosolic protein that may dislocate proteins from the ER and target multi-ubiquitinated substrates to the proteasome (Ye et al., 2001; Jarosch et al., 2002; Rabinovich et al., 2002). Unlike pαF, ubiquitination is necessary for the degradation of CPY*, and several components of the ubiquitin-conjugating machinery have been implicated in CPY* proteolysis: the ubiquitin-conjugating enzymes Ubc6p and Ubc7p (Hiller et al., 1996); Der3p/Hrd1p, a membrane-anchored ubiquitin-protein ligase (E3) (Bays et al., 2001; Deak and Wolf, 2001); and Cue1p, which recruits Ubc7p to the ER membrane (Biederer et al., 1997).

An integral membrane protein shown to be an ERAD substrate in both yeast and mammalian cells is the cystic fibrosis transmembrane conductance regulator (CFTR) (Yang et al., 1993; Pind et al., 1994; Jensen et al., 1995; Ward et al., 1995; Zhang et al., 2001). Mutations in CFTR that prevent its maturation in the ER and subsequent transport lead to cystic fibrosis. Like CPY*, CFTR degradation requires ubiquitination (Ward et al., 1995; Jensen et al., 1995; Zhang et al., 2001), but unlike soluble substrates there is no requirement for calnexin or BiP (Zhang et al., 2001). By contrast, Ssa1p facilitates CFTR degradation (Zhang et al., 2001).

In the event that ERAD is unable to rid the secretory pathway of aberrant polypeptides the unfolded protein response (UPR) may be activated. The UPR is present in all eukaryotic cells and increases the ability of the ER to tolerate misfolded proteins (for a review, see Kaufman, 1999; Ng et al., 2000). Because the UPR and ERAD provide complementary facets of secretory protein 'quality control', it is not surprising that ERAD and the UPR are functionally intertwined. For example, the scope of the UPR was examined by microarray analysis on addition of the *N*-linked glycosylation inhibitor, tunicamycin, and during accumulation of the mouse major histocompatibility complex class I heavy chain (H-2K^b), a substrate for ERAD when unassembled (Ploegh et al., 1979; Hughes et al., 1997; Casagrande et al., 2000). When H-2K^b was overexpressed in yeast, mRNAs encoding several chaperones and known UPR targets were upregulated three- to seven-fold, but many uncharacterized genes were also induced. Travers et al. (Travers et al., 2000) determined the transcriptional scope of the yeast UPR using tunicamycin and dithiothreitol (DTT), which prevents disulfide bond formation, and again, known UPR-target genes and uncharacterized open reading frames (ORFs) were induced. In both screens, UPR-target genes included those required for ERAD. Moreover, yeast lacking nonessential components of both the ERAD and UPR pathways exhibit synthetic growth defects, suggesting that the two pathways function in concert (Travers et al., 2000; Ng et al., 2000; Friedlander et al., 2000).

Given the fact that many genes induced by the UPR are uncharacterized ORFs and that a subset of known UPR targets encode ERAD-requiring proteins, we selected 69 ORFs upregulated by the UPR. Mutants deleted for the corresponding genes were then screened for A1PiZ degradation deficiencies (*add*). From these analyses, six *ADD* gene products were identified. Furthermore, analysis of CFTR, pαF and CPY* degradation in the new *add* mutants underscores the diverse requirements for the removal of individual ERAD substrates and points to the complexity with which this pathway functions.

Materials and Methods

Materials, strains, plasmids

Deletion mutant yeast strains (Table 1) constructed in *S. cerevisiae* strain BY4742 (*MATα his3Δ1 leu2Δ0 lysΔ0 ura3Δ0*) (Winzeler et al., 1999) were purchased from Research Genetics (Huntsville, AL). Wild-type (*MAT**a** ura3-52 leu2-3,122 ade2-101*) and *kar2-1* mutant yeast (*MAT**a** ura3-52 leu2-3,122 ade2-101 kar2-1*) were previously described (Brodsky et al., 1999). Electrocompetent *Escherichia coli* strain HB101 (*F$^-$ mcrB mrr hsdS20(r$_B^-$, m$_B^-$) recA13 supE44 ara14 galK2 lacY1 proA2 rpsL20(Sm^r) xyl5 λ$^-$ leu mtl*) was purchased from Life Technologies (Rockville, MD). Plasmid pYES.2.0 (2 μm, Amp^r, *URA3*) containing the cDNA sequence for either the A1PiM wild-type form or the A1PiZ mutant form under control of the galactose (*GAL1*) inducible promoter were previously described (McCracken and Kruse, 1993). The expression vectors p415.ADH (*CEN6/ARSH4*, Amp^r, *LEU2*, *ADH1* alcohol dehydrogenase I constitutive promoter) and p425.CYC1 (2 μm, Amp^r, *LEU2*, *CYC1* constitutive promoter and translation termination) were purchased from American Type Culture Collection (Rockville, MD). The hemagglutin (HA)-tagged CPY*

Table 1. Deletion strains screened for A1PiZ stabilization

Strain number	Gene	Description	H-2K^{b}*	TuniT‡	A1PiZ§
01	YBR201W	*DER1*, degradation in the ER	3.10	23.65	102
02	YCL044C	Unknown	3.42	7.36	–
03	YDL110C	Unknown	3.05	0.93	–
04	YDR210W	Unknown	3.39	0.08	–
05	YDR256C	*CTA1*, catalase A	4.02	5.88	–
06	YDR400W	*URH1*, uridine nucleosidase	3.30	2.50	128
07	YEL060C	*PRB1*, vacuolar protease B	3.19	0.54	–
08	YER091C	*MET6*, homocysteine methyltransferase	3.84	–3.60	–
09	YGR044C	*RME1*, regulator of meiosis	3.22	–0.90	–
10	YHR044C	*DOG1*, 2-deoxyglucose-6-phosphatase	3.29	11.67	–
11	YIL015W	*BAR1*, alpha factor protease	3.09	0.52	–
12	YIL090W	Unknown	4.38	2.45	–
13	YKL073W	*LHS1*, lumenal Hsp70	5.24	5.37	–
14	YKL106W	*AAT1*, aspartate aminotransferase	3.13	5.66	–
15	YKL163W	*PIR3*, cell wall structural protein	4.00	0.16	–
16	YLR104W	Unknown	3.41	6.99	–
17	YLR205C	*HMX1*, presumptive haem oxygenase	3.17	–0.51	–
18	YLR423C	*APG17*, essential for autophagy	3.00	5.13	–
19	YLR429W	*CRN1*, coronin	3.01	4.05	–
20	YML054C	*CYB2*, cytochrome B2	4.25	0.70	–
21	YMR040W	Unknown	7.43	5.33	–
22	YMR315W	Unknown	3.31	3.07	–
23	YMR316W	*DIA1*, represses invasive growth	4.38	–0.20	–
24	YNL173C	*MDG1*, multicopy suppressor of defective G-protein	3.33	–1.73	–
25	YNL274C	Unknown	3.01	–1.75	–
26	YNR075W	*COS10*	3.04	1.99	–
27	YOL016C	*CMK2*, calmodulin-dependent protein kinase	3.22	–0.50	–
28	YOL019W	Unknown	3.60	3.43	–
29	YOL031C	Unknown	5.56	7.80	–
30	YOR264W	*DSE3*, daughter specific expression 3	4.03	2.44	–
31	YOR317W	*FAA1*, long chain fatty acyl:CoA synthetase	3.16	1.10	–
32	YOR385W	Unknown	3.66	–3.79	–
33	YPL187W	*MF(ALPHA)1*, mating factor alpha	10.1	0.42	–
34	YPL256C	*CLN2*, G1/S cyclin	3.09	2.90	–
35	YPR119W	*CLB2*, G2/M cyclin	3.01	–1.38	–
36	YML033W	*SRC1*	1.20	11.031	–
37	YMR184W	Unknown	4.63	10.708	130
38	YMR264W	*CUE1*, Ubc7p binding and recruitment protein	1.14	0.852	100
39	YHR043C	*DOG2*, 2-deoxyglucose-6-phosphatase	2.63	11.452	126
40	YJL073W	*JEN1*, DnaJ-like protein	2.21	11.066	–
41	YIL005W	*EPS1*, protein disulfide isomerase	1.29	3.188	105
42	YOR321W	*PMT3*, dolichyl phosphate-D-mannose: protein *O*-D-mannosyltransferase	2.52	9.834	–
43	YOL013C	*HRD1/DER3*, ubiquitin-protein ligase	1.88	3.636	106
44	YHR129C	*ARP1*, actin-related protein	1.42	9.968	–
45	YOR099W	*KTR1*, alpha-1,2-mannosyltransferase	2.14	9.822	–
46	YOR129C	Unknown	0.95	8.406	–
47	YOR288C	*MPD1*, protein disulfide isomerase	6.03	13.434	–
48	YBR050C	*REG2*, regulatory subunit of (PP1)Glc7	1.51	8.648	–
49	YBR176W	*ECM31*, 3-methyl-2-oxobutanoate hydroxymethyltransferase	1.31	9.314	–
50	YBR224W	Unknown	0.56-1.07	9.471	–
51	YBR246W	Unknown	1.42	10.243	–
52	YCL008C	*STP22*, Ste pseudorevertant	1.20	8.137	–
53	YCL047C	Unknown	1.72	9.211	–
54	YDR295C	*PLO2*, ploidy related	1.59	9.723	–
55	YDL073W	Unknown	0.78-1.09	8.702	–
56	YDL125C	*HNT1*, histidine triad protein	2.67	10.387	–
57	YDR008C	Unknown	0.82	10.351	–
58	YLR126C	Unknown	2.21	12.172	–
59	YDR411C	Unknown	2.38	10.017	–
60	YDR488C	*PAC11*, dynein intermediate chain	1.08	9.026	–
61	YDR491C	*HRD3*	0.66	–0.74	ND¶
62	YGL014W	*PUF4*, Pumilio-homology domain protein	1.48	27.20	–
63	YGR010W	Unknown	2.07	8.034	–
64	YGR037C	*ACB1*, acyl-CoA-binding protein	3.38	8.241	–
65	YGR062C	*COX18*, cytochrome c oxidase	1.52	9.337	–
66	YKL206C	Unknown	2.50	9.804	150
67	YLR380W	*CSR1*, *SFH2*, phosphatidylinositol transfer protein	1.67	8.373	136
68	YFL049W	Unknown	1.41	26.781	130
69	YFR020W	Unknown	1.92	24.215	–
70	YFR026C	Unknown	1.50	10.051	–
71	YFR041C	Unknown	2.46	14.266	–
72	YGR284C	*ERV29*, ER-Golgi transport vesicle protein	2.73	8.618	127
73	YAL005C	*SSA1*, cytosolic Hsp70	1.84	–1.473	104

*The H-2K^{b} value is equal to the fold increase of mRNA level in response to H-2K^{b} accumulation as reported by Casagrande et al. (Casagrande et al., 2000).

‡The TuniT value is equal to the fold increase of mRNA level in response to tunicamycin exposure as reported by Travers et al. (Travers et al., 2000).

§The percentage of A1PiZ accumulated in each deletion strain compared with that in WT cell line was determined by densitometric analysis of immunoassay results. The percentage values of A1PiZ accumulated in each *add* strain, as well as each deletion mutant of genes required for other ERAD substrates, are listed. For all other deletion mutants that display WT A1PiZ accumulation values, the percentage of A1PiZ is designated.

¶The deletion mutant did not grow in galactose, and thus could not be evaluated using the assay.

expression vector (*CEN/ARS*, Amp^r, *URA3*) was provided by Davis Ng (Pennsylvania State University, PA) (Ng et al., 2000), and pJC104, to measure induction of the UPR, was contributed by Peter Walter (University of California, San Francisco, CA) (Cox et al., 1993). Antibodies used include: rabbit anti-human A1Pi (Dako, Carpenteria, CA), horseradish peroxidase-conjugated goat anti-rabbit (United States Biochemical, Cleveland, OH), monoclonal mouse anti-HA (Roche Molecular Biochemicals, Indianapolis, IN) and sheep anti-mouse immunoglobulin G (IgG) horseradish-peroxidase-conjugated antibody (Amersham Pharmacia Biotech, Piscataway, NJ).

Construction of double mutants

Each newly identified *add* mutant strain was mated to *kar2-1* yeast (see above) using established methods (Kaiser et al., 1994). Sporulation in the selected diploids was induced by nitrogen starvation and the spores were dissected and analyzed as described by Adams et al. (Adams et al., 1997) to determine their genotypes.

Cloning and expression of genes

Genomic DNA was isolated from the BY4742 wild-type parent strain as described by Hoffman and Winston (Hoffman and Winston, 1987). WT *ADD* gene sequences were amplified from genomic DNA by PCR using oligonucleotides (Life Technologies) specific for each locus with alterations to introduce unique restriction endonuclease cleavage sites immediately 5′ of the ATG or ~300 base pairs upstream of the ATG, and unique restriction endonuclease cleavage sites 3′ of the stop codon. Primer sequences used are available on request. Wild-type *ADD* genes lacking a promoter (i.e. those immediately 5′ of the ATG) were inserted into the p415.ADH vector, and each wild-type gene containing a putative promoter sequence (i.e. those containing ~300 base pairs 5′ of the ATG) was inserted into the p415.CYC1term vector. The correct insertion of each gene was determined by automatic DNA sequence analysis (following standard protocols) using primers specific for the p415.ADH or p425.CYC1term vectors. The p415CYC1term vector was generated by removing the *CYC1* promoter from p415.CYC1 with a *Sac*I/*Bam*HI digest, creation of blunt ends and ligation so that the multiple cloning region (MCR) retained the *Bam*HI through *Xho*I restriction sites preceding the *CYC1* termination sequence.

Yeast and *E. coli* transformation

Yeast transformation was carried out by a standard lithium acetate procedure (Gietz and Woods, 1994), and transformants were isolated after growth in selective medium containing 2% dextrose. The Cell-Porator *E. coli* Pulser (GibcoBRL, Series 1613, Rockville, MD) was used to electroporate HB101. Plasmids were isolated from bacterial transformants using the Quantum Prep Plasmid Miniprep kit (Bio-Rad, Hercules, CA).

Authentication of BY4742 mutants

Genomic DNA was isolated from BY4742 mutant strains: *add06*, *add37*, *add39*, *add66*, *add67* and *add68*, as described by Hoffman and Winston (Hoffman and Winston, 1987). A kanamycin cassette for each corresponding *ADD* locus was amplified from genomic DNA by PCR using oligonucleotides (Life Technologies) as described by Research Genetic's published deletion module PCR strategy (Wach et al., 1994). Upstream 45 bp and downstream 45 bp sequences and the kanamycin gene were verified by automatic DNA sequence analysis (following standard protocols) using primers specific for each locus.

Colony-blot immunoassay

The colony-blot immunoassay was a modification of a previously described procedure (McCracken et al., 1996). Three microliters of 0.001 OD/μl of overnight cell cultures were spotted onto a nitrocellulose disc overlaid on medium containing 2% galactose to induce expression of A1Pi (M or Z) followed by incubation at 35°C for 36 hours. Cells were lysed and blots developed as described by McCracken et al. (McCracken et al., 1996). The density of A1Pi at each colony spot was quantified using Molecular Analyst.

Radiolabeling, immunoprecipitation and phosphorimaging

To assay A1PiZ degradation, yeast were grown at 30°C in selective medium containing 2% galactose to induce A1Pi expression for 24 hours before analysis. Using a protocol described by Brodsky et al. (Brodsky et al., 1998), 13 OD_{600} cells were pulsed with ^{35}S-Easy Tag (NEN) for 20 minutes and chased with 10× cold cysteine/methionine mix. Samples were taken at the indicated time points. Cell lysis and immunoprecipitation were performed as described (Brodsky et al., 1998). Proteins were resolved by 10% SDS-PAGE and visualized using a BioRad PhosphorImager (Hercules, CA). Quantification was performed using Molecular Analyst. CPY* degradation was measured similarly by pulse-chase radiolabeling and immunoprecipitation as previously published (Zhang et al., 2001), starting with HA-tagged CPY* expression vector (see above) transformed cells and using anti-HA antibody.

CFTR degradation assay

Yeast expressing CFTR were grown to an OD_{600} of 0.5 at 30°C before cycloheximide was added to make a final concentration of 100 μg/ml. The cells were incubated at 30°C with shaking and 0.5 OD units were harvested at the indicated time points. Total protein in the lysates (10 μl of 100 μl final volume) (Zhang et al., 2001) were resolved by SDS-PAGE, followed by immunoblot analysis using monoclonal mouse anti-HA (Roche Molecular Biochemicals), sheep anti-mouse IgG horseradish peroxidase-conjugated antibody (Amersham Pharmacia Biotech), SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL), and visualizing using a BioRad PhosphorImager. Quantification was performed using Molecular Analyst.

Assays for ERAD and UPR induction

Yeast ER-derived microsomes and cytosol were prepared and the in vitro ERAD assay was performed as described by McCracken and Brodsky (McCracken and Brodsky, 1996). Quantification of the resulting phosphorimaged gels was performed using Molecular Analyst. UPR induction was measured after growth of each transformed strain in selective medium to log phase (OD_{600}=~1). Cell extracts were prepared by agitation of washed cells with glass beads, and β-galactosidase activity was measured using published protocols (Adams et al., 1997).

Database searches

NCBI BLASTN 2.2.3 (Altschul et al., 1990; Karlin and Altschul, 1990; Karlin and Altschul, 1993; Tatusova and Madden, 1999) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), the PSORT WWW Server (Nakai, 1991; Nakai, 2000) (http://psort.nibb.ac.jp/) and reports on the random and systematic epitope-tagging of ORFs in the yeast genome (Ross-Macdonald et al., 1999) (http://ygac.med.yale.edu/ygac-cgi/front_page_OE.html) were used to identify and predict cellular locations of the Add proteins. BLASTP 2.2.3 generated multiple protein sequence alignments and sequence relatedness data. The Add06p inosine-uridine hydrolase family signature was identified using The Swiss Institute of Bioinformatics ScanProsite (http://www.expasy.ch/tools/scanprosite/). Signal peptide cleavage sites were identified by Center for Biological Sequence Analysis SignalP V1.1 (Nielsen et al., 1997)

(http://www.cbs.dtu.dk/services/SignalP/). PSORT WWW Server identified N- and C-terminal ER retention signals. Structural homology predictions were made using the 3D-PSSM Web Server V.2.6.0 (Fischer et al., 1999; Kelley et al., 1999; Kelley et al., 2000) (http://www.sbg.bio.ic.ac.uk/~3dpssm/).

Results

Selection of candidate ERAD genes

Because the UPR upregulates genes required for ERAD (Travers et al., 2000; Casagrande et al., 2000) it was likely that at least a subset of the uncharacterized UPR-target genes would also encode components required for ERAD. To investigate this hypothesis, we selected uncharacterized genes from the pool of UPR-targets identified by Casagrande et al. (Casagrande et al., 2000) in which the UPR was induced by the accumulation of the ERAD substrate H-2K^b, and identified by Travers et al. (Travers et al., 2000) in which tunicamycin was used to induce the UPR. An arbitrary cut-off of a 3.0-fold increase in message in response to H-2K^b accumulation (deletion mutants #1-35) or an 8.0-fold increase in message in response to tunicamycin exposure (deletion mutants #36, 37, 39, 40, 42 and 44-60, 62-72) was used to compile a list of candidate genes (Table 1). Known ERAD genes that are not UPR-targets were also included in the screen (#38, 41, 43, 61 and 72; see below).

A1PiZ accumulation in mutants deleted for UPR-target genes

To determine whether yeast lacking the UPR-target genes were ERAD-defective, 73 deletion mutants were transformed with a galactose-inducible A1PiZ expression vector and tested for A1PiZ degradation deficiencies (*add* mutant phenotype) (McCracken et al., 1996) using a colony-blot immunoassay (see Materials and Methods). On the basis of the knowledge that A1PiZ accumulates in ERAD-defective cells (McCracken et al., 1996; Werner et al., 1996), strains exhibiting an *add* mutant phenotype should contain an increased amount of immunoreactive A1PiZ under steady-state conditions and thus display a 'spot' of greater intensity on the colony-blot compared with wild-type (WT) cells (Fig. 1). Controls for this assay were WT cells carrying the expression vector lacking an A1Pi gene, which resulted in background intensity, WT cells expressing but degrading A1PiZ, which produced a low-intensity spot, and WT cells expressing A1PiM, a stable protein that accumulates in the yeast ER and thus displays a high-intensity spot (McCracken et al., 1996). Of the uncharacterized yeast mutants examined in this screen, six (*add06*, *add37*, *add39*, *add66*, *add67* and *add68*) were putative *add* mutants after initial screening and re-screening (Table 2, Fig. 1). The amount of A1PiZ accumulated reproducibly in these mutants was >125% of that in the WT parent strain.

Two genes upregulated by the UPR and known to be involved in ERAD (#1 – *DER1*, and #72 – *ERV29*) (Plemper et al., 1997; Caldwell et al., 2001) were included in our analyses, along with known ERAD genes that are not UPR-induced (#38 – *CUE1*, #41 – *EPS1*, and #43 – *HRD1/DER3*, *#61-HRD3*, *#73-SSA1*) (Lenk and Sommer, 2000; Wang and Chang, 1999; Hampton and Bhakta,1997; Plemper et al., 1999; Zhang et al., 2001). Interestingly, with the exception of *ERV29*, these deletion mutants displayed a WT *ADD* phenotype (Table 1, Fig. 1), indicating that the proteins encoded by the genes are not essential for the degradation of A1PiZ.

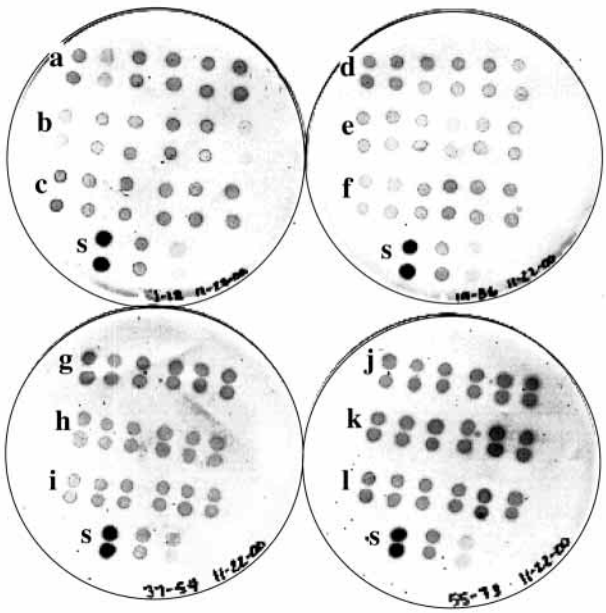

Fig. 1. New *add* mutants identified by colony-blot immunoassay screening. Representative immunoassay performed on each gene deletion strain as described in Materials and Methods. Rows (a-l) show, in duplicate from left to right, colonies of deletion mutants #01-06 (a), 07-12 (b), 13-18 (c), 19-24 (d), 25-30 (e), 31-36 (f), 37-42 (g), 43-48 (h), 49-54 (i), 55-60 (j), 62-67 (k), 68-73 (l). Row (s) shows duplicates of the M, Z and O parent strain standard controls from left to right, expressing wild-type A1PiM or mutant A1PiZ, or the pYES2.0 vector with no gene insertion. Those deletion mutants exhibiting a darker colony spot than that of the BY4742 WT parent expressing A1PiZ were selected as putative ERAD mutants and re-assayed to eliminate false-positive results. Only deletion mutants 06, 37, 39, 66, 67, 68 and 72 displayed a consistent signal darker than the BY4742 WT parent (Z) in subsequent colony-blot immunoassays. For example, deletion mutants 59 and 60 were selected from this assay but were eliminated by at least two negative results in future assays.

To authenticate the identity of the deleted genes in each mutant that we obtained commercially, the polymerase chain reaction (PCR) was used with primers corresponding to the insertion site of the deletion modules (Wach et al., 1994), and the products were sequenced (Materials and Methods). We thus confirmed the identity of the deleted genes (data not shown).

The rate of A1PiZ degradation is decreased in *ADD* deletion mutants

To verify the mutant phenotype in *add06*, *add37*, *add39*, *add66*, *add67* and *add68*, we performed pulse-chase analyses to monitor A1PiZ degradation. As anticipated, A1PiZ was stabilized in each *add* deletion mutant; ~40-50% of A1PiZ was degraded in the deletion mutants at 60 minutes compared with 60% degraded in the WT parent strain (Fig. 2). This level of A1PiZ stabilization was similar to that seen in temperature-sensitive *kar2* strains (Brodsky et al., 1999) and previously described *ADD* mutant strains (McCracken et al., 1996).

Table 2. Bioinformatic predictions for *ADD* gene products

Deletion mutant	Gene	Gene product*†	Cellular location*‡	Sequence similarity*‡§	Structural similarity¶	Interactions**
add06	YDR400w	Urh1p – uridine nucleoside N-ribohydrolase	Cytoplasm	Inosine-uridine hydrolase family signature aa 49-59	Purine nucleoside hydrolases	Yih1p
add37	YMR184w	Unknown	Cytoplasm	25%/45% of 85 aa†† Jac1p, 23%/54% of 86 aa Gos1p, 27%/44% of 90 aa Uso1p, 52%/63% of 17 aa Sec13p	Ubiquitin-conjugating enzyme (Ubc4p)	TFIIB
add39	YHR043c	Dog2p – 2-deoxyglucose-6-phosphate phosphatase	Cytoplasm	88%/92% of 245 aa Dog1p, 35%/51% of 227 aa Rhr2p, 35%/50% of 215 aa Hor2p	Haloacid dehalogenase-like hydrolases	Apg17p, Cdc12p
add66	YKL206c	Unknown	Cytoplasm	Signal peptide cleavage site	EF-hand containing calmodulin-like protein	Pre1p, Arl3p
add67	YLR380w	Csr1p/Sfh2p – phosphatidylinositol transfer protein; Sec14p bypass	Cytoplasm; ER-membrane associated	N-terminal -XXRR and C-terminal -KKXX ER retention signals; 29%/44% of 229 aa Sec14p	Phosphatidylinositol transfer protein (Sec14p)	
add68	YFL049w	Unknown	Nucleus/cytoplasm	29%/44% of 176 aa Npl6p	Heme-linked catalase; N-terminal nucleophile aminohydrolases (proteasome α subunit)	Ydr370cp, Fre7p, Snf5p

*NCBI BLAST.
†Li et al., 2000.
‡PSORTII.
§ScanProsite; SignalP.
¶3D-PSSM.
**Uetz et al., 2000; Ito et al., 2001; Ho et al., 2002; Gavin et al., 2002.
††Percentage identity/percentage similarity between proteins across a specific number of amino acids.

To complement the ERAD defects observed in the *ADD* deletion strains, WT copies of *ADD06*, *ADD37*, *ADD39*, *ADD66*, *ADD67* and *ADD68* were cloned into the p415.ADH constitutive expression vector and were transformed into the corresponding strains. However, only three strains (*add66*, *add67* and *add68*) displayed complementation of the *add* phenotype. Next, the *ADD* genes were cloned along with their native promoters into the p425.CYC1term vector. For this analysis, the putative promoters were assumed to be located within 300 base pairs upstream from the ATG translation start site. We then assayed the *ADD* deletion strains expressing the appropriate WT gene from their native promoters and found that all strains, except *add67*, showed complementation of the *add* phenotype (Fig. 3). The *add67* strain showed complementation only when the *ADD67* gene was expressed from the p415.ADH vector (Fig. 3). We note that the various promoters used for these complementation analyses direct expression at different levels; however, it is also possible that positional effects might influence expression from native promoters when inserted into the corresponding plasmids. Because complementation of the mutant phenotype is seen when WT *ADD* genes are expressed from specific promoters and not others, we speculate that the level of expression of these *ADD* genes may be crucial for their function in *ERAD*. In support of this hypothesis, Lenk et al. (Lenk et al., 2002) reported that the level of expression of the yeast ubiquitin-conjugating enzyme Ubc6p influenced ERAD activity.

The degradation of other ERAD substrates is compromised in the *add* mutants

Because of the diverse requirements for the degradation of ERAD substrates (see Introduction), we examined pαF, CPY* and CFTR degradation in each *add* mutant strain and in a WT parent. We discovered that none of the new *add* mutants strongly affected the rate of CPY* degradation as determined by pulse-chase analysis (data not shown), whereas CPY* degradation was attenuated in *kar2-1* mutant yeast that express an ERAD-defective form of BiP (Brodsky et al., 1999; Zhang et al., 2001). In accordance with these data, Caldwell et al. (Caldwell et al., 2001) previously analyzed yeast deleted for YKL206c and YMR184w, which encode Add66p and Add37p, respectively, and reported that the mutant strains degraded CPY* with WT efficiency.

To examine whether the degradation of an ER membrane protein is affected in these *add* mutants, we next measured CFTR stability using a cycloheximide chase protocol (Materials and Methods). CFTR was stabilized to varying extents when compared with the WT parent strain (Fig. 4). The *add06* and *add39* deletion mutants displayed the greatest degradation defects; 10% and 17% of CFTR, respectively, was degraded at 20 minutes, compared with 35% CFTR degradation observed in the WT parent.

Finally, we assayed pαF degradation in vitro using cytosol and ER-derived microsome fractions prepared from deletion mutants and WT cells. In principle, this assay should permit us

to define the compartment(s) in which the corresponding proteins normally function. However, it is possible that cytosolic factors can contaminate membrane fractions, that the requirement for gene products can be bypassed in vitro and/or that the action of regulators may be obviated in defined, in vitro systems. Nevertheless, we found that both cytosol and microsomes from *add37*, *add39*, *add66* and *add67* supported the degradation of pαF, suggesting that the gene products were not required for pαF degradation in vitro. However, reactions with *add06* or *add68* microsomes and cytosol displayed a noticeable degradation defect; 46% of pαF was proteolyzed at 40 minutes compared with 68% proteolysis in WT reactions (Fig. 5A). Although the stabilization of pαF with both *add06* and *add68* cytosol was significantly greater than that seen with WT cytosol, the difference in the rate of degradation between the WT and the mutant strain was not as great as that observed with other strains that stabilize pαF. For example, WT *KAR2* microsomes and cytosol proteolyzed 64% of pαF in 40 minutes, whereas the reaction with *kar2-1* cytosol and microsomes showed only 35% pαF degradation (Brodsky et al., 1999). Additionally, when studying the role of calnexin in ERAD we observed 80% degradation of pαF using materials prepared from the *CNE1* parent, whereas 55% proteolysis was apparent when reagents from the *cne1* delete were used (McCracken and Brodsky, 1996). Thus, the WT parents of the various mutant strains show variations in the efficiency of pαF degradation in vitro, indicating that strain variation and perhaps proteasome activity in the cytosol preparations influence the extent of pαF proteolysis in this assay.

Next, pαF degradation was assayed using microsomes and cytosol from WT and *add06* (Fig. 5B) or *add68* (Fig. 5C) strains in all combinations. Only reactions containing mutant cytosol with either WT or mutant microsomes exhibited a decreased rate of pαF degradation similar to that seen in Fig. 5A. These results suggest that both Add06p and Add68p function in the cytosol to facilitate pαF degradation. In accordance with this hypothesis, the Add68 and Add06 proteins lack putative signal sequences, and Add06p tagged at the C-terminus was shown to reside in the yeast cytoplasm/nucleus (Ross-Macdonald et al., 1999).

The unfolded protein response is modestly enhanced in *add66* and *add67* yeast

ERAD defects can lead to induction of the UPR because aberrant proteins accumulate in the ER (reviewed by Fewell et al., 2001). By contrast, more subtle or substrate-specific ERAD defects might not induce the UPR. To examine UPR levels in the *add* mutants, a UPR reporter plasmid containing four repeats of the UPR element (UPRE) 5′ to the β-galactosidase-encoding gene (pJC104) (see Cox et al., 1993) was transformed into each strain. As a control, isogenic wild type and *kar2-1* mutant yeast were also transformed with pJC104. Cells were grown at 30°C to log phase, cell extracts were prepared and β-galactosidase activity was measured. We found that the *kar2-1* strain exhibited an ~11-fold increase in the UPR compared with the wild type; this is consistent with the fact that all soluble ERAD substrates examined in this mutant are stabilized and that protein folding is compromised in this strain (Fig. 6) (Simons et al., 1995; Brodsky et al., 1999; Zhang et al., 2001). We also noted that UPR induction in the *add* strains varied considerably relative to the *ADD* parent. For example, *add06*, *37*, *39*, 68 displayed either no increase or a modest decrease in the UPR, whereas *add66* and *add67* exhibited a 3-4-fold induction. These data suggest that the Add66 and Add67 proteins may be required more generally for ERAD and/or that the lack of these proteins affects ER physiology. By contrast,

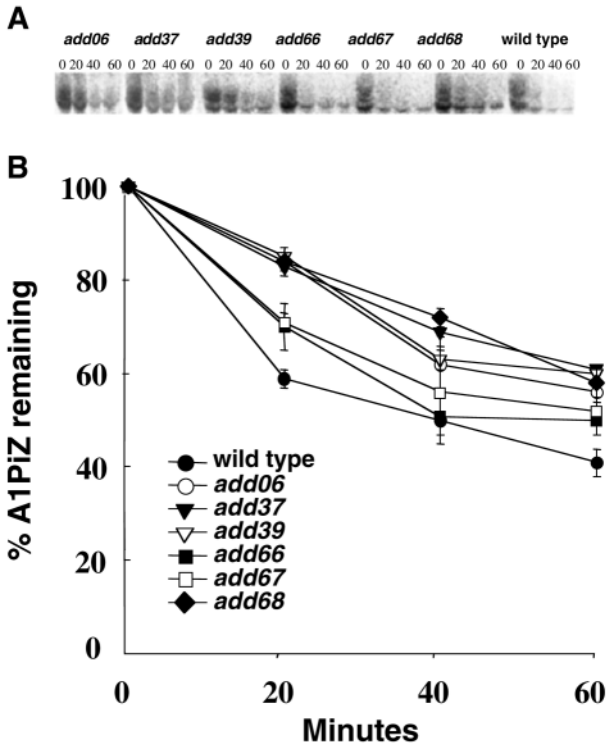

Fig. 2. The A1PiZ degradation rate is slowed in the *add* mutants. Pulse-chase radiolabeling experiments were performed with *add* mutants and the parent wild type, BY4742, expressing A1PiZ. (A) A1PiZ was immunoprecipitated from the cell extracts at 0, 20, 40 and 60 minutes and resolved on a 10% SDS-PAGE (Methods and Materials). (B) The relative amounts of A1PiZ were determined using the Bio-Rad Phosphor Analyses program with the amount of A1PiZ at the zero time point set at 100%. Results shown are the average of five independent experiments, ±s.d.

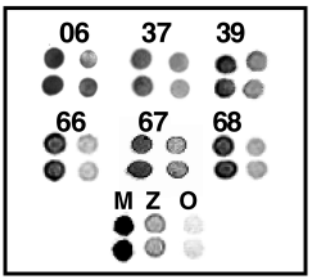

Fig. 3. Complementation of the *add* phenotype. Representative immunoassays performed as described in Materials and Methods. Deletion mutants (*add06*, *add37*, *add39*, *add66*, *add67* and *add68*) expressing A1PiZ were transformed with expression vectors carrying the corresponding *ADD* gene (right) or with the appropriate vector without gene insert (left) and duplicate colonies were assayed. Standard controls were BY4742 WT parent stain expressing A1Pi (M), mutant A1PiZ (Z) or the vector with no gene insertion (0).

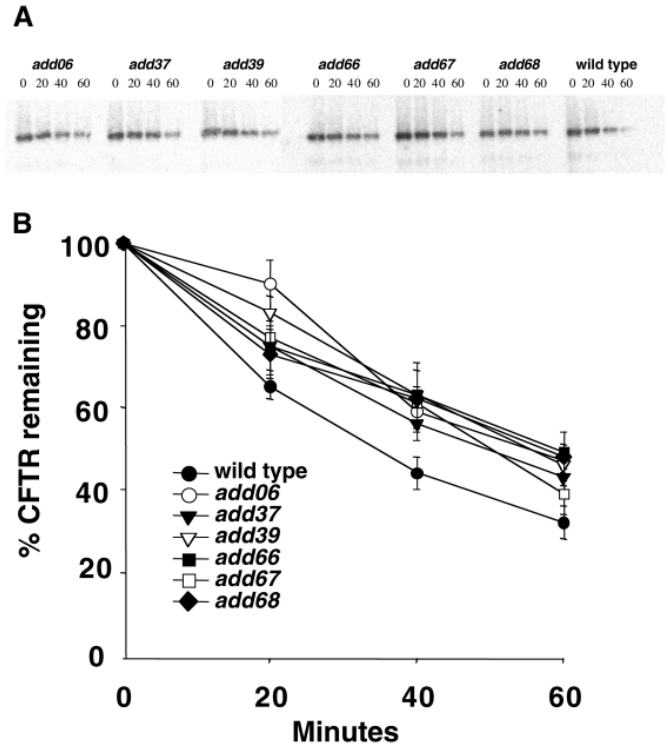

Fig. 4. CFTR degradation is compromised in the *add* mutants. CFTR-expressing cells were grown to mid-log phase, cycloheximide was added, and the cells were harvested at the indicated time points. (A) Cell extracts were prepared at 0, 20, 40 and 60 minutes and subjected to SDS-PAGE followed by quantitative immunoblot analysis. (B) The relative amounts of CFTR were determined using the Bio-Rad Phosphor Analyses program with the amount of CFTR at the zero time point set at 100%. Results shown are the average of three independent experiments, ±s.d.

cells might be able to compensate for loss of the proteins encoded by the other *add* mutants, or the absence of these gene products might lead to a defect in the degradation of only select proteins.

The *ADD68* deletion strain is hypersensitive to cadmium

Addition of cadmium to living cells causes oxidative damage to cellular components, catalyzes protein unfolding, induces the expression of heat-shock proteins in yeast and is toxic at elevated concentrations (Jungmann et al., 1993). Furthermore, compromising the cellular ubiquitin-proteasome pathway by mutation or expression of dominant-negative mutants leads to hypersensitivity to cadmium (Tsirigotis et al., 2001). Thus, hypersensitivity to cadmium may be indicative of defects in proteasome-mediated degradation of aberrant proteins.

To determine whether the *ADD* mutants were sensitive to cadmium, exponentially growing cells were spotted in tenfold serial dilutions on plates containing 15 μM or 30 μM cadmium chloride ($CdCl_2$) and incubated at either 30°C or 37°C for 3 days. All strains tested showed mild sensitivity to cadmium, as indicated by slowed growth, and one strain, *add68*, was hypersensitive to cadmium (Fig. 7). The effect of 30 μM $CdCl_2$ was marginally greater than that of 15 μM $CdCl_2$, and a slight

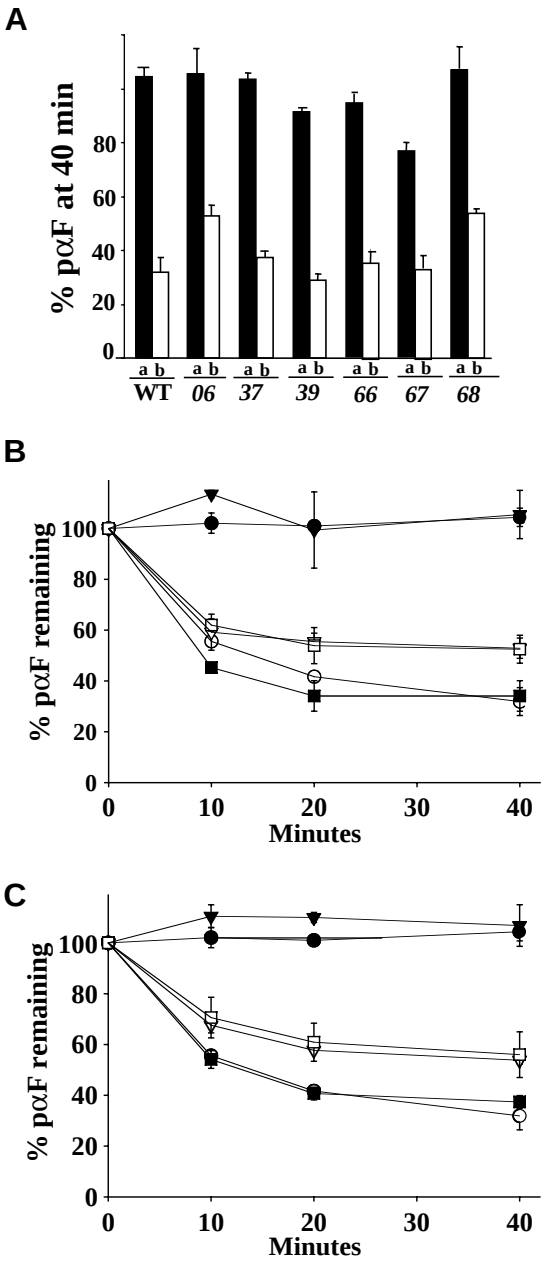

Fig. 5. Microsomes and cytosol prepared from *add37*, *add39*, *add66* and *add67*, but neither *add06* nor *add68* are proficient for pαF degradation in vitro. (A) Microsomes containing pαF were prepared from each *ADD* deletion strain and incubated in the (a) absence or (b) presence of cytosol made from the same strain; the amount of pαF was determined at 0 and 40 minutes after addition of cytosol or buffer. (B,C) Microsomes and cytosol were prepared from both WT and deletion strains (B, *add06* and C, *add68*) and analyzed for ERAD in vitro in the indicated combinations of microsomes/cytosol: ○ WT/WT; ● WT/buffer; ▲ *add*/buffer; △ *add*/*add*; □ WT/*add*; ■ *add*/WT. The amount of pαF was determined using the Bio-Rad Phosphor Analyses program. Data represents the mean of triplicate experiments, ±s.d.

increase in sensitivity was seen at 37°C (data not shown). One control strain, *add72*, deleted for the gene ERV29 that is required for the HIP pathway of ERAD (Haynes et al., 2002),

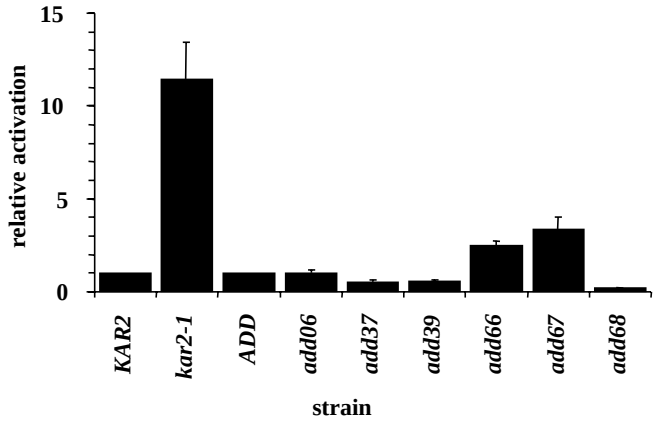

Fig. 6. UPR induction in *add* mutants. Respective isogenic wild type and the *kar2-1* and *add* mutant strains were transformed with the UPR reporter plasmid, and relative activity (compared to the wild type) was assayed as described in the Materials and Methods. Data represent the means of two experiments, each performed with two unique transformants of each strain.

displayed sensitivity to cadmium. Interestingly, a strain deleted for the *HRD1/DER3* gene (*add43*) that encodes an ubiquitin-protein ligase (Bays et al., 2001) was not hypersensitive to $CdCl_2$, indicating that defective ERAD does not necessitate cadmium hypersensitivity.

Yeast exhibit a growth defect when *ADD37* deletion is combined with the *kar2*-1 allele

To determine the growth phenotypes of strains lacking the newly identified ADD genes and a known ERAD-requiring gene, add mutant strains were mated to *kar2-1* yeast, an ERAD-specific mutant allele of BiP. Previous results indicated that yeast containing this allele and deleted for the ER lumenal chaperone, calnexin (*CNE1*), exhibited poorer growth than cells either lacking calnexin or containing *kar2-1* alone (Brodsky et al., 1999). We first found that *add66* and *add68* mutant cells exhibit severe sporulation defects; thus, only *kar2-1* progeny combined with *add06, add37, add39* and *add67* could be obtained by this method. Next, we noted that only *add37* yeast exhibited a synthetic growth defect at 37°C when combined with the *kar2-1* allele (Fig. 8).

Bioinformatic analysis of the *ADD* gene products

To gain insight into the protein product of each *ADD* gene and their possible roles in ERAD, sequence homology and structural analysis searches were performed. This information is summarized in Table 2, and we present models for how the corresponding gene products might facilitate ERAD in the Discussion, below.

Discussion

We have used a simple assay to rapidly and successfully screen 68 UPR-target genes in search of uncharacterized components of the ERAD pathway. The six genes identified are required for maximal A1PiZ degradation and exhibit diverse effects on the degradation of other substrates. These results suggest that

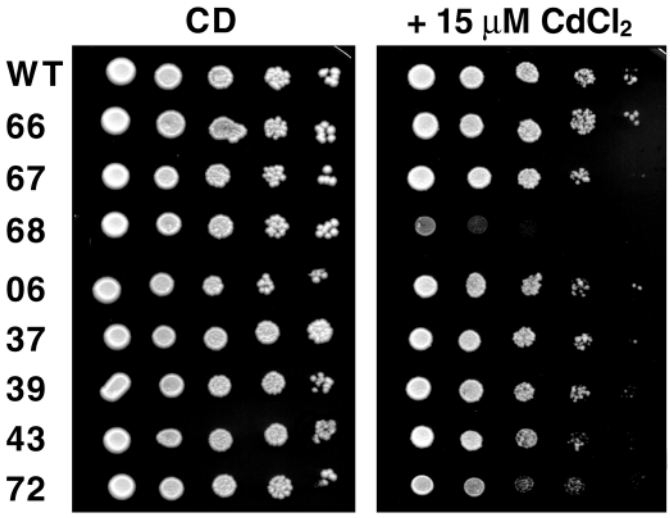

Fig. 7. Cells deleted for *ADD68* are hypersensitive to cadmium. Exponentially growing liquid cultures of each *ADD* deletion strain (66, 67, 68, 06, 37, 39, 43 and 72) and the isogenic parent (WT) were spotted in ten-fold dilutions of 0.001 OD/μl on complete medium (CD) or medium containing cadmium (+15 μM $CdCl_2$) and were incubated at 30°C for 36 hours. Plates were imaged using a Mustek 600-11-CD scanner.

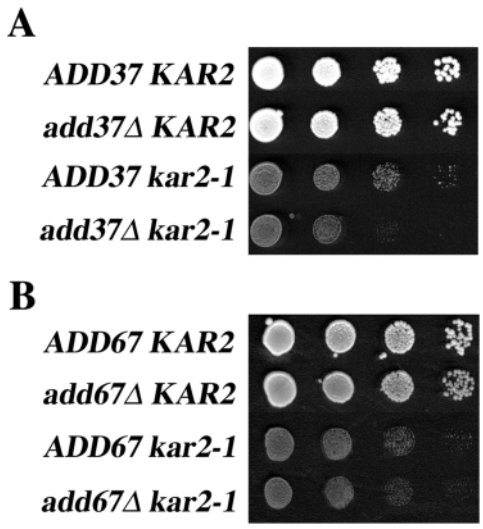

Fig. 8. *kar2-1 add37* double mutants display a synthetic growth defect. Exponentially growing cultures of dissected tetrads were plated at tenfold dilutions on complete media and incubated at 37°C for two days. Progeny from crosses *add37Δ X kar2-1* (A) and *add67Δ X kar2-1* (B) were ordered in rows from top to bottom: WT strain, *ADD/KAR2*; ADD deletion mutant strain, *addΔ/KAR2*; temperature-sensitive *kar2-1* strain, *ADD/kar2-1;* and the double mutant, *addΔ/kar2-1*. The results for crosses *add06Δ X kar2-1* and *add39Δ X kar2-1* were similar to that seen for *add67Δ X kar2-1*.

ERAD is comprised of several overlapping pathways, on which the action of many gene products impact.

The concept that ERAD may be comprised of multiple pathways is supported by the diverse requirements for the degradation of ERAD substrates (Table 3) and by their diversity in structures and post-translational modifications. For

example, most, but not all, ERAD substrates are ubiquitinated before their degradation, and most, but not all, are glycosylated (reviewed by Fewell et al., 2001). These features will play a profound role in dictating which partners a given ERAD substrate will encounter en route to its degradation. Also, ERAD substrates are either integral membrane or soluble proteins in the ER, and differences in the chaperone requirements for the degradation of these two classes of substrates have led us and others to propose distinct mechanisms for their removal (see below). Finally, we suspect that the degree to which a protein misfolds to become an ERAD substrate may be quite varied. Some ERAD substrates probably attain little, if any, secondary structure in the ER, whereas others at least partially fold and become glycosylated and disulfide-bonded before their retrotranslocation and degradation. For A1PiZ, this issue is particularly pertinent as it is highly aggregation prone (Lomas et al., 1992). Thus, gene products that are required for the degradation of A1PiZ may be specialized, catalyzing the degradation and maintaining the solubility of such aggregation-prone ERAD substrates. In the future, it will be vital to understand whether the *add* mutants – such as those identified in this study – affect the solubility of A1PiZ in the ER and/or whether they are directly involved in its degradation.

Our results also serve as an important starting point to develop hypotheses regarding the functions of these *ADD* gene products during ERAD. Although striking sequence identities were not apparent between the *ADD* genes and other characterized genes, each *ADD* gene contains motifs that suggest specific functions. Such suggested homologies will drive our future research efforts. For example, the *ADD06* gene (YDR400w) encodes a 378 amino acid residue uridine nucleoside *N*-ribohydrolase (Urh1p) (Table 2). ScanProsite analysis of the amino acid sequence indicates a nucleoside hydrolase family signature at residues 49-59 and previous studies have demonstrated its role in hydrolyzing nucleosides (Magni et al., 1975; Kurtz et al., 1999). More relevant, however, may be the observation that Add06p interacts with Yih1p (Uetz et al., 2000), a protein involved in protein synthesis regulation during stress (Kubota et al., 2000). These data suggest that defects in stress-induced regulation might impact ERAD. Consistent with this notion, cell stress was recently shown to regulate ERAD (VanSlyke and Musil, 2002).

YMR184w, the gene deleted in *add37*, encodes a cytosolic protein of unknown function. BLAST2 sequence alignments show that the region of Add37p spanning amino acids 105 and 186 is 25% identical and 45% similar to the mitochondrial J-type chaperone Jac1p, which is involved in the assembly of iron sulfur clusters (Lutz et al., 2001; Voisine et al., 2001). However, these sequence homologies occur outside of the J domain of Jac1p. Potentially more relevant, the region between amino acids 99 and 180 displays 23% identity and 54% similarity with Gos1p, a v-SNARE protein (McNew et al., 1998). Moreover, the region of Add37p spanning amino acids 95 and 170 is 27% identical and 44% similar to Uso1p, a protein necessary for ER to Golgi protein transport (Nakajima et al., 1991), and the amino acid region 62 to 78 displays 52% identity and 63% similarity with Sec13p, a component of the COPII vesicle coat (Salama et al., 1997). Intriguingly, 3D-PSSM predicted the Add37p to structurally resemble the ubiquitin-conjugating enzyme, Ubc4p. Although it is hard to reconcile these data into a working model for Add37p function in ERAD, it is tempting to speculate that Add37 is required for protein secretion and that A1PiZ degradation, like three other soluble ERAD substrates, requires a functional ER to Golgi pathway (Caldwell et al., 2001; Vashist et al., 2001). In accordance with this model, we found that deletion of the gene encoding Erv29p, which is a soluble cargo receptor in the ER (Belden and Barlowe, 2001), inhibits AiPiZ degradation (Fig. 1).

The *ADD67* gene (YLR380w) is an endocytic membrane/vesicle-associated phosphatidylinositol transferase known as Csr1p/Sfh2p (Li et al., 2000; Cockcrost and De Matteis, 2001). The region spanning amino acid 109 and 316 of Csr1p/Sfh2p displays 29% identity and 44% similarity with Sec14p and like Sec14p appears to function in ER-to-Golgi vesicle transport (Li et al., 2000), suggesting another possible link between the *ADD* gene products and the secretory pathway. An alternative scenario is that lipid composition is altered in the *csr1* mutant, leading to an ERAD-defect. Pertinent to this view is the recent finding that lipid rafts may be important to sort proteins that have a potential to become ERAD substrates if mutated (Bagnat et al., 2001).

The *ADD66* gene (YKL206c) encodes a cytosolic protein that has been found to be associated with an ADP-ribosylation factor-like protein known as Arl3p, and with Pre1p, a proteasome subunit (Ho et al., 2002). Two models can be envisioned for Add66p function. First, the protein may associate with Pre1p and be involved in proteasome assembly

Table 3. Components required (R) and not required (N) for the ER-associated degradation of specific substrates in yeast

	ERAD substrate			
Component	A1PiZ	pαF	CFTR	CPY*
Proteasome	R	R	R	R
Sec61p		R		R‡
BiP	R	R	N	R
Calnexin		R	N	
Protein disulfide isomerase		R		N§
Cer1p/Lhs1p/Ssi1p	**N**	N¶		N¶
Ssa1p	**N**	N	R	
Der1p	**N**			R
Der3p/Hrd1p	**N**		N**	R
Hrd3p				R‡‡
Ubc6/7p	N§§	N	R	R¶¶
Cue1p	**N**			R
Erv29p	**R**			R
Erv14p				R
Urh1p (Add06p)	**R**	**R**	**R**	**N**
Add37p	**R**	**N**	**R**	**N**
Dog2p (Add39p)	**R**	**N**	**R**	**N**
Add66p	**R**	**N**	**R**	**N**
Csr1p/Sfh2p (Add67p)	**R**	**N**	**R**	**N**
Add68p	**R**	**R**	**R**	**N**

Citations included in text except where indicated. Results of the current study are in bold.

‡Plemper et al., 1997.

§Gillece et al., 1999.

¶Nishikawa et al., 2001.

**Zhang et al., 2001.

‡‡Plemper et al., 1999.

§§Werner et al., 1996.

¶¶Hiller et al., 1996.

or function. Second, because *arl3* mutants exhibit defects in secretion (Huang et al., 1999), the *add66* mutation may similarly be compromised for ERAD because of defects in ER-to-Golgi trafficking (see above). Because of these strong connections to components/pathways that impact upon ERAD, it may not be surprising that deletion of the *ADD66* gene induces the UPR (Fig. 7).

YHR043c encodes a 2-deoxyglucose-6-phosphate phosphatase (Add39p/Dog2p), a cytoplasmic protein involved in carbohydrate metabolism. The entire Dog2p sequence displays 88% identity and 92% similarity to Dog1p, the region spanning amino acids 6 and 231 of Dog2p is 35% identical and 51% similar to Rhr2p, and amino acids 6 to 219 of Dog2p display 35% identity and 50% similarity to Hor2p, all of which are phosphatases. Yeast two-hybrid assays have shown interactions between Dog2p and Apg17p, a protein involved in autophagy (Kamada et al., 2000; Ito et al., 2001). Perlmutter and colleagues have found that mutant A1Pi accumulation leads to the propagation of autophagocytic vesicles in mammalian cells (Teckman and Perlmutter, 2000), suggesting that this ERAD substrate may be degraded by the vacuole/lysosome when the ERAD machinery is overwhelmed. Thus, *add39* yeast might be compromised for autophagocytosis, leading to an increased demand on the ERAD pathway.

The *ADD68* gene (YFL049w) is predicted to encode a nuclear membrane protein. The region spanning amino acids 50 and 220 displays 29% identity and 44% similarity with Npl6p, a protein isolated originally in a screen for nuclear protein localization mutants (Nelson et al., 1993). Intriguingly, another gene isolated from that screen, Npl4p, associates with Cdc48p and with a third protein, Ufd1, that together are required for the ERAD of several substrates (Bays and Hampton, 2002; Tsai et al., 2002). An additional link between Add68p and ERAD comes from structural predictions that Add68p resembles an N-terminal nucleophile aminohydrolase, such as the proteasome α subunit protein. Like the proteasome (Enenkel et al., 1998), Add68p can be found associated with the ER and in the cytosol (Ross-Macdonald et al., 1999) and appears to function in the cytosol (Fig. 5). Moreover, the *ADD68* deletion mutant is sensitive to cadmium, a phenotypic characteristic of cells with defective proteasome activity. Overall, as with the other new *ADD* gene products, further work is clearly needed to elucidate, at the molecular level, how these factors facilitate ERAD.

In summary, the results of our screen and the timeliness of available bioinformatic/genome-wide analyses in yeast have provided us with many testable hypotheses about the functional roles for these *ADD* gene products in ERAD. Our results provide further insight into the complexity of ERAD, and underscore the importance of analyzing additional ERAD substrates and in defining the genetic requirements for their degradation. A biochemical characterization of the *ADD* gene products will also lead to a better, more complete understanding of the complexity of the substrate specific requirements for ERAD. However, we note that our search was limited to those genes that are not essential for cell viability. Thus, microarray analysis of differential gene expression using cells over-expressing A1PiZ should identify essential and other nonessential genes required for the degradation of A1PiZ and will further help elucidate the molecular mechanism of the degradation of this important ERAD substrate. Finally, and more generally, our screening approach may be applicable to the analysis of any ERAD substrate in yeast for which antiserum is available.

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