Degradation signals for ubiquitin system proteolysis in *Saccharomyces cerevisiae*

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Combinations of different ubiquitin-conjugating (Ubc) enzymes and other factors constitute subsidiary pathways of the ubiquitin system, each of which ubiquitinates a specific subset of proteins. There is evidence that certain sequence elements or structural motifs of target proteins are degradation signals which mark them for ubiquitination by a particular branch of the ubiquitin system and for subsequent degradation. Our aim was to devise a way of searching systematically for degradation signals and to determine to which ubiquitin system subpathways they direct the proteins. We have constructed two reporter gene libraries based on the *lacZ* or *URA3* genes which, in *Saccharomyces cerevisiae*, express fusion proteins with a wide variety of C-terminal extensions. From these, we have isolated clones producing unstable fusion proteins which are stabilized in various *ubc* mutants. Among these are 10 clones whose products are stabilized in *ubc6*, *ubc7* or *ubc6ubc7* double mutants. The C-terminal extensions of these clones, which vary in length from 16 to 50 amino acid residues, are presumed to contain degradation signals channeling proteins for degradation via the *UBC6* and/or *UBC7* subpathways of the ubiquitin system. Some of these C-terminal tails share similar sequence motifs, and a feature common to almost all of these sequences is a highly hydrophobic region such as is usually located inside globular proteins or inserted into membranes.

Keywords: degradation signals/proteolysis/*UBC6/UBC7*/ubiquitin

Introduction

Proteolysis of specific regulatory proteins such as cyclins, cyclin kinase inhibitors, transcriptional regulators and membrane receptors plays an important role in many cellular processes including metabolic control, cell cycle progression and signal transduction (reviewed in Ciechanover, 1994; Hochstrasser, 1995, 1996a). There is extensive evidence that defined sequence elements or structural features of proteins constitute degradation signals which confer instability by channeling them into specific degradation pathways. Such degradation signals have been recognized in bacteria, yeast and higher eukaryotes (Gottesman and Maurizi, 1992; Varshavsky, 1992;

Ciechanover, 1994; Hochstrasser, 1995; Hilt and Wolf, 1996; Keiler *et al.*, 1996; Rechsteiner and Rogers, 1996).

In eukaryotic cells, most unstable proteins are degraded by the ubiquitin system which tags them for degradation by ligating ubiquitin to them (reviewed in Hershko and Ciechanover, 1992; Hochstrasser, 1996b). The ubiquitin-tagged molecules usually are degraded by the 26S proteasome (Jentsch and Schlenker, 1995; Rubin and Finley, 1995; Hilt and Wolf, 1996) although ubiquitination of some membrane proteins marks them for endocytosis and degradation in the vacuole (Hochstrasser, 1996a). Attachment of ubiquitin to proteins involves a series of enzymatic steps. Ubiquitin-activating enzymes (E1 or Uba) form a high energy thiol ester bond with the C-terminus of ubiquitin which subsequently is transferred to the thiol of a ubiquitin-conjugating enzyme (E2 or Ubc). Finally, ubiquitin is transferred from the Ubc to the target protein with or without the assistance of a ubiquitin protein ligase (E3). Usually, repeated cycles of ubiquitination attach a multiubiquitin chain to the target protein. In *Saccharomyces cerevisiae*, there are 13 Ubc's which are assisted by an unknown number of E3 enzymes and/or other ancillary factors. Additional factors may also be required to modify the target protein or to activate the ubiquitination system prior to ubiquitin ligation (Varshavsky, 1992; Lahav-Baratz *et al.*, 1995; King *et al.*, 1996; Lanker *et al.*, 1996).

Combinations of Ubc's with each other and with various E3s and additional factors determine the specificity for the ubiquitination and degradation of specific target proteins. Sometimes more than one Ubc is involved in the degradation of a single protein and even in the recognition of a single signal (Chen *et al.*, 1993; Sommer and Jentsch, 1993; Biederer *et al.*, 1996; Cox and Walter, 1996). Although an enormous number of combinations of Ubc's with each other, with E3s and with other factors are possible, which could recognize a wide variety of target motifs, few degradation signals or destabilizing structural features of proteins are known and even fewer have been analyzed in detail (reviewed in Ciechanover, 1994; Hochstrasser, 1995; Hilt and Wolf, 1996). Well-defined sequences which confer instability in yeast include destabilizing N-terminal amino acid residues (N-end rule; Varshavsky, 1992), the cyclin B destruction box (King *et al.*, 1996) and the C-terminal signal which is required for ubiquitination, endocytosis and vacuolar degradation of the Ste2 receptor (Hicke and Reizman, 1996). Proteolysis of the Hac1p transcription factor is contingent on the presence of a 10 amino acid residue C-terminal signal which is exchangeable with a stabilizing sequence by alternative splicing regulated by the unfolded protein response (Cox and Walter, 1996). Longer polypeptide stretches such as the 67 residue Deg1 region of Mat α 2 (Chen *et al.*, 1993) and a non-removable N-terminally

fused ubiquitin (Johnson *et al.*, 1995) also destabilize proteins. In addition to these well-defined signals, several more general sequence or structural motifs have been associated with protein instability. PEST sequences (enriched in Pro, Glu, Ser, Thr and Asp) are found in many unstable proteins and have been shown, for specific proteins, to be required for degradation both via the ubiquitin–proteasome system and by the proteasome without ubiquitination (Kornitzer *et al.*, 1994; Yaglom *et al.*, 1995; Rechsteiner and Rogers, 1996). In fusion proteins carrying artificial signals, short hydrophobic stretches and sequences predicted to form an amphipathic α -helix were found to destabilize (Sadis *et al.*, 1995).

There is extensive evidence that non-native states of proteins caused by mutation, denaturation or amino acid analog substitution lead to their accelerated degradation (Parag *et al.*, 1987). How such abnormal proteins are recognized and earmarked for destruction is still not clear, but one possibility is that cryptic degradation signals are exposed by partial unfolding or by failure of subunits to oligomerize (Sommer and Jentsch, 1993; Biederer *et al.*, 1996). Aberrant endoplasmic reticulum (ER) proteins are transported selectively to the cytoplasm and degraded by the proteasome via a process involving the Sec61p translocon and Ubc6p plus Ubc7p (Hiller *et al.*, 1996; Biederer *et al.*, 1997; Plemper *et al.*, 1997).

Although the findings outlined above support the view that particular subsets of ubiquitin system components must recognize specific signals, much remains to be learnt about the way in which recognition and ubiquitination are achieved in different pathways. One reason for the gaps in knowledge about degradation signals and their corresponding pathways is that no systematic approach has been used to discover them. We have therefore developed yeast vectors and screening methods to search systematically for novel protein degradation signals. For this purpose, we have made yeast expression vector libraries producing β -galactosidase and Ura3p C-terminal fusion proteins with diverse C-terminal appendages. Rapidly degraded fusion proteins can be isolated at a high frequency by this new method and their C-terminal sequences determined and studied. The ubiquitin–proteasome system subpathways involved in the degradation of specific proteins can also be identified easily. C-terminal appendages channeling proteins for degradation via a pathway involving the ubiquitin-conjugating enzymes Ubc6p and Ubc7p are described here.

Results

Use of β -galactosidase and Ura3p fusion protein libraries to isolate ubiquitin system targeting signals

Two types of vector were used to prepare C-terminal fusion protein libraries to identify signals targeting proteins for degradation via the ubiquitin system. One type of yeast vector (pBRR; Figure 1A) was designed to make β -galactosidase C-terminal fusion proteins, the second to produce Ura3p C-terminal fusion proteins (pOC9; Figure 1B). Vectors generating C-terminal fusion proteins were chosen because only a single in-frame fusion is necessary to obtain chimeras of β -galactosidase or Ura3p with portions of proteins expressed *in vivo*. Both vectors

Fig. 1. *LacZ* and *URA3* vectors used to prepare libraries. (A) pBRR88 yeast expression vector has the *LacZ* gene with a C-terminal multiple cloning site derived from the bacterial pUR288 vector (Rüther and Müller-Hill, 1983). pBRR78 and pBRR89 (not shown) are identical except that they have a C-terminal multiple cloning site of pUR278 and pUR289, respectively. Each vector produces fusions in a different reading frame. (B) pOC9 vector is derived from the *TRP1* yeast vector pRS414 (Sikorski and Hieter, 1989) carrying *URA3* under a *CUP1* promoter. It produces Ura3p with a C-terminal HA epitope. MCS1 and MCS2 are multiple cloning sites derived from the pRS414 vector. In all cases, genomic *Sau3A*I fragments were cloned into the C-terminal *Bam*HI site of the vectors.

can be used to identify targeting signals channeling fusion proteins for degradation via various Ubc subpathways. Here we report on the use of libraries derived from these vectors for identifying signals channeling proteins for degradation via Ubc6p and Ubc7p.

The *lacZ* library of ~100 000 clones was made by inserting yeast genomic DNA fragments into a C-terminal cloning site attached to a *lacZ* gene in a 2μ yeast expression vector. The screening method for unstable β -galactosidase fusion proteins was based on that used in previous studies, with a vector producing N-terminally modified β -galactosidase, to isolate the N-end rule E3 gene *UBR1* (Bartel *et al.*, 1990). Yeast cells bearing a plasmid expressing a stable enzymatically active β -galactosidase fusion protein form blue colonies on X-gal plates. On the other hand, cells with a plasmid producing an unstable β -galactosidase fusion protein form white colonies. White colonies may also be formed for other reasons such as folding problems, low enzymatic activity or impaired synthesis of β -galactosidase due to the C-terminal extension. Clones making unstable proteins can be distinguished from other clones derived from white colonies because they form blue colonies in mutants which inhibit the degradation of their product.

Fig. 2. β -galactosidase-specific activities of ubiquitin-proteasome system mutants carrying the β -galactosidase-SL17 fusion protein vector. Hatched bars, mutant; full bars, isogenic strain with unmutated gene. All the mutants except *pre1-1pre2-1* and *pre1-1pre4-1* were null mutants. Data are expressed as a percentage of data from a wild-type control carrying a parent vector expressing β -galactosidase without a C-terminal tail.

Since the yield of clones producing unstable fusion proteins from the *lacZ* library was low, we devised an alternative approach based on Ura3p fusion proteins. Previous work has shown that Ura3p can be destabilized by fusing to its N-terminus the Deg1 degradation signal of Mat α 2 or unstable variants of dihydrofolate reductase (Chen *et al.*, 1993; Dohmen *et al.*, 1994). We constructed a yeast expression vector (pOC9) with a *CUP1* promoter-controlled *URA3* gene fused to a C-terminal hemagglutinin (HA) epitope followed by a cloning site (Figure 1B). Insertion of yeast genomic DNA fragments into this cloning site produces a variety of Ura3p C-terminal fusion proteins. A library containing ~50 000 clones with inserts was made in this way. Clones giving low Ura3p activity in a wild-type background, which were 5-fluoro-orotic (5-FOA) acid resistant but unable to grow without uracil, were first selected. From these, we selected clones which, in a *ubc6ubc7* double mutant background, supported growth without uracil but did not support growth on 5-FOA. Many of these clones produced unstable Ura3p fusion proteins which were stabilized in the *ubc6ubc7* mutant.

An unstable β -galactosidase fusion protein stabilized in the *ubc6ubc7* mutant

The SL17 plasmid, isolated from the *lacZ* library, forms white colonies on X-gal plates in a wild-type background but blue colonies in a *ubc6ubc7* mutant background. As an indication of the stabilization of the β -galactosidase-SL17 fusion protein in various ubiquitin-proteasome system mutants, we measured the steady-state activity of β -galactosidase (Figure 2). Single *ubc6* or *ubc7* null mutations and *ubc6ubc7* double mutations markedly elevated β -galactosidase-specific activity. There was little difference in the effect of the *ubc6ubc7* double mutants and that of each of the two single mutants, indicating an overlapping function of the two Ubcs. The findings indicated that the SL17 fusion protein is degraded by the ubiquitin system with the participation of Ubc6p and Ubc7p. β -galactosidase activity was also strongly elevated in the *pre1pre2* proteasome mutant (Heinemeyer *et al.*, 1993) and in the *doa4* deubiquitinating enzyme mutant

Fig. 3. Pulse-chase analysis of β -galactosidase-SL17 fusion protein degradation. (A) Ubiquitin ladders (arrows) formed by β -galactosidase-SL17 fusion protein in wild-type (SUB62) but not in *ubc6ubc7* double null mutant (SS414) cells. (B) β -galactosidase-SL17 fusion protein degradation in the wild-type strain and in the *ubc6ubc7* double null mutant. (C) β -galactosidase-SL17 fusion protein degradation in the wild-type strain (WCG4a) and the *pre1-1pre2-1* mutant (WCG4a-11/12). The arrow shows the degradation intermediate.

which indirectly impairs proteasome function (Papa and Hochstrasser, 1993) (Figure 2). These observations point to the involvement of the proteasome in SL17 fusion protein degradation. Other mutant backgrounds shown in Figure 2 had no significant effect on β -galactosidase activity. Also tested without detectable effect were the following mutants: *ubc3* (*cdc34*), *ubc9*, *ubc10* and *ubc11* (not shown).

In pulse-labeling experiments, the β -galactosidase-SL17 fusion protein formed ubiquitin ladders in a wild-type background but not in a *ubc6ubc7* mutant background (Figure 3A). This implies that Ubc6p and Ubc7p are an essential part of the recognition machinery for the SL17 signal.

Pulse-chase experiments showed that the β -galactosidase-SL17 fusion protein was degraded rapidly in a wild-type background. In a *ubc6ubc7* double mutant background (Figure 3B), the β -galactosidase-SL17 fusion protein was stabilized, though not completely. The β -galactosidase-SL17 fusion protein was completely stabilized in a *pre1-1pre2* proteasome mutant background (Figure 3C), indicating the involvement of the proteasome in its degradation. The partial stabilization of the SL17 fusion protein in the *ubc6ubc7* mutant, in contrast to its complete stabilization in the *pre1pre2* mutant, suggests that unknown degradation pathways in addition to that involving Ubc6p or Ubc7p may be involved in its proteolysis.

A degradation intermediate of the β -galactosidase-SL17 fusion protein (apparent mol. wt ~97 kDa) accumulated and subsequently was broken down more slowly than the

Fig. 4. Evidence that the intermediate of β -galactosidase-SL17 degradation is derived from its N-terminus. Wild-type cells carried a plasmid (HAL-SL17) producing a β -galactosidase-SL17 fusion protein with an N-terminal HA epitope. Pulse-chase analysis was performed with an antibody against β -galactosidase (recognizing an epitope between residues 650 and 926) and with an anti-HA epitope antigen (which recognizes only the N-terminus of the fusion protein). The arrow shows the degradation intermediate.

original fusion protein (Figure 3B and C). The question arose as to whether the 97 kDa intermediate is formed by the removal of a piece of the 120 kDa fusion protein at the C- or the N-terminus. To determine this, we constructed a plasmid expressing a β -galactosidase-SL17 fusion protein with an HA epitope at its N-terminus. In a pulse-chase experiment (Figure 4), the fate of the fusion protein was followed with an antibody against β -galactosidase, recognizing an epitope between residues 650 and 926, and with an antibody against the HA epitope which recognizes only the N-terminus. Since the intermediate reacted with both antibodies, it must be derived from the N-terminal portion of the molecule. In studies on the degradation of N-end rule substrates such as K- β -gal, R- β -gal, L- β -gal and Y- β -gal, a long-lived intermediate of ~90 kDa was formed (Bachmair *et al.*, 1986). It is not clear whether these differences in β -galactosidase processing stem from the location of the degradation signal, from the position of the ubiquitinated lysine residues, from the nature of the signal and the Ubcs involved or from a combination of these factors.

The SL17 C-terminal tail confers instability on Ura3p

The 50 amino acid residue SL17 tail was attached to the C-terminus of Ura3p by inserting its coding sequence into the pOC9 vector. Wild-type cells carrying the vector expressing this fusion protein were unable to grow on medium without uracil and grew on 5-FOA, indicating low Ura3p activity (Figure 5A). The same vector in a *ubc6ubc7* mutant background supported growth without uracil but not on 5-FOA, suggesting stabilization of the Ura3p-SL17 fusion protein in the mutant background. Pulse-chase analysis (Figure 5B) showed that the Ura3p-SL17 fusion protein was so unstable in a wild-type background that it was not detectable even at t_0 of the pulse-chase experiments. In a *ubc6ubc7* mutant background, the fusion protein was clearly visible (Figure 5B). Thus the tail of the β -galactosidase-SL17 fusion protein can be transferred from one protein to another, retaining its destabilization characteristics, suggesting that it acts as an autonomous signal.

Isolation of Ura3p fusion proteins stabilized in a *ubc6ubc7* background

Nine distinct clones producing Ura3p fusion proteins which were unstable in wild-type cells and stabilized in *ubc6ubc7* mutants were found by pulse-chase analysis. The fusion proteins are unstable to different degrees in

Fig. 5. Attachment of the SL17 C-terminal extension destabilizes Ura3p. The sequence encoding the SL17 C-terminal extension was transferred to the pOC9 vector to express a Ura3p-SL17 fusion protein. (A) Plating of *ubc6ubc7* double null mutants (SS414) and wild-type cells (SUB62) carrying pOC9-SL17 on 5-FOA or on minimal medium with or without uracil. (B) Pulse-chase analysis of Ura3p-SL17 fusion protein degradation in the *ubc6ubc7* mutant and the wild-type background. Arrow shows fusion protein.

Fig. 6. Pulse-chase analysis of degradation of unstable Ura3p fusion proteins which are stabilized in a *ubc6ubc7* background. For details about clones see Table I.

wild-type cells (Figure 6). Some of the fusion proteins are so unstable that the protein is not seen (CL9, CL10 and CL12) or seen with difficulty (CL2 and CL15) even at t_0 of pulse-chase experiments. Many of the Ura3p fusion proteins are completely stabilized in a *ubc6ubc7* background but others (SL17, CL9 and CL12) are only

Table I. Deduced C-terminal destabilizing sequences

CL1	ACKNW FSSLS HFVIHL
CL2	SLISLPLPTRV KFSSLL LIRIMKIITMTFPPKKLRS
CL6	FYYPIWFARVLLVHYQ
CL9	SNP FSS LGASLLIDSVSLKSNWDTSSS CLISFSSVM FSSTTRS
CL10	CRQRFSCHLTASYPQSTVTPFLAFLRRDFFFLRHNSAD
CL11	GAPHVVLDFELRITNPLSHIQSVSLQITLIFCSLPSLILSKFLQV
CL12	NTPLFSKSFSTTCGVAKKTL LAQISS LFLLSSNIAV
CL15	PTVKNSPKIF CL SSPYLAFNLEYLS RIFST LSKCSNTLLTSLS
CL16	SNQLKRLWLWLLEVRSFDRTLRRPWHLPS
SL17	SISFVIRSHASIRMGASNDFFHKLYFTK CLTSVILSKFLI HLRLRSTPRV

Underline = hydrophobic region in the hydropathy plot (Kyte and Doolittle, 1982); window size = 7; bold = FSSL or similar motif.

partially stabilized, suggesting the participation of additional unidentified pathways in their degradation.

Table I shows the sequences of the C-terminal tails of the unstable fusion proteins. Appendage lengths are short, varying from 16 to 50 amino acid residues. None are parts of known or putative open reading frames (ORFs) in the yeast database. Comparison of the tail sequences with ORFs in the yeast database did not reveal any striking homologies. It is not yet clear if it is significant that all the C-terminal appendages isolated so far are short (<50 amino acid residues) and are not apparently parts of natural proteins. There may be selection against the longer C-terminal appendages expected from fusions in-frame with *in vivo* protein-coding regions. For example, a bulky protein appendage would be more likely than a small one to interfere with folding, subunit assembly or activity of the β -galactosidase or Ura3p. However, only one out of six random insertions can be in-frame, and 70% of the yeast genome consists of ORFs (Goffeau *et al.*, 1996). Thus, at best, only one out of nine fusions can be expected to produce a tail with an *in vivo* ORF. Since we have so far isolated only 10 unstable fusion proteins degraded via Ubc6p plus Ubc7p, it is premature to conclude that there is a bias in the selection against longer stretches which are parts of protein-coding regions.

Most of the C-terminal appendages have a high percentage of serine plus threonine residues. This percentage is 1.7 times higher than that computed for the translation in all six reading frames of the complete sequences of yeast chromosomes 1, 2, 3 and 4. Hydropathy plots (Kyte and Doolittle, 1982) show that all the tails have a strongly hydrophobic region such as is usually buried inside globular protein molecules or inserted into membranes. A frequently recurring motif, sometimes found twice in the same sequence, is FSSL or its variants (defined more generally as [bulky hydrophobic]–[S or T]–[S or T]–[bulky hydrophobic]) (Table I, bold). Sequence analysis according to Altuvia *et al.* (1994) reveals that CL1, CL2 and CL12 have a similar region including their FSSL-like sequence and extending downstream from it which can be generalized as [F,W or Y]–[S or T]–[S or T]–[I,L or V]–XX–[hydrophobic]–X–[I,L or V]–[H-bond acceptor]. CL9, CL11, CL15 and SL17 also have slight variants of this motif. Amphipathic α -helices have been suggested previously to constitute a signal for Ubc6p plus Ubc7p pathway degradation (Sadis *et al.*, 1995). However, few of the C-terminal appendages in Table I have sequences predicted to form amphipathic α -helices.

Fig. 7. Pulse-chase analysis of fusion proteins with mutated or synthetic degradation signals. (A) Effect of deletions of the C-terminal extension of the β -galactosidase-SL17 fusion protein: 1–50, complete SL17 extension; 1–43, SL17 extension minus 10 C-terminal residues followed by RRL contributed by the vector; 1–30, SL17 extension minus 20 C-terminal amino acid residues. (B) Pulse-chase analysis of the complete CL1 extension or of partial sequences, as shown, attached to the C-terminus of HA-tagged Ura3p. Deletions and synthetic signals were made by PCR. An exception was the SL17 1–43 construct which was made by excising a *Sau*3A1 fragment and inserting it into the vector.

Deletion analysis of destabilizing sequences

Deletion of 10 C-terminal amino acid residues from the 50 residue β -galactosidase-SL17 fusion protein does not alter its instability (Figure 7A). However, removal of 20 C-terminal amino acid residues completely stabilizes the fusion protein. Thus destabilization depends on 10 amino acid residues within the highly hydrophobic region of the SL17 extension.

Removal of HFVIHL from the 16 residue C-terminal tail of the CL1 fusion protein stabilizes it (Figure 7B). Thus, the FSSL sequence on its own is insufficient to confer instability. This conclusion is supported by the finding that attachment of the sequence FSSLA directly to the HA epitope-tagged Ura3p produced by the vector does not destabilize it (Figure 7B). Attachment of FSSLSHFVIHL, which has the extended consensus sequence described above, is also insufficient to destabilize Ura3p-HA (Figure 7B). Thus, the degradation signal must extend over most, if not all, of the CL1 appendage.

The destabilizing C-terminal amino acid residues of the SL17 fusion protein and the CL1 C-terminal appendage have similar sequences which can be defined as [F or V]–[S or T]–S–[V or L]–...–S[H or K]–F–[V or L]–IHL. Failure of the CL1 version of this sequence to destabilize Ura3-HA shows that it is necessary but not sufficient for destabilization.

Discussion

The β -galactosidase and Ura3p fusion protein libraries described above have enabled us to identify a number of C-terminal appendages which promote the degradation of proteins via Ubc6p plus Ubc7p. Although many of the C-terminal elements in Table I have similar sequence elements, it is possible that more general molecular features rather than a specific consensus sequence may be involved in recognition by the Ubc6p/Ubc7p pair and its associated factors. A feature common to almost all of the destabilizing C-terminal extensions in Table I is a strongly hydrophobic region (Kyte and Doolittle, 1982). Such exposed hydrophobic stretches may be recognized by the ubiquitination system as regions normally located inside globular protein molecules or buried in membranes. An indication that such a recognition mechanism might operate comes from studies on the Ubc6p plus Ubc7p-dependent degradation of mutants of the membrane translocation proteins Sec61p and Sss1p. When Sec61p is mutated, its interaction with an associated protein, Sss1p, is weakened and both proteins become unstable (Sommer and Jentsch, 1993; Esnault *et al.*, 1994; Biederer *et al.*, 1996).

Almost all of the hydrophobic stretches of the signals in Table I have charged residues embedded in them or adjacent to them. In this respect, they resemble the C-terminal signals involved in ER protein quality control via retrograde transport into the cytoplasm and degradation by the proteasome (Bonifacino *et al.*, 1990, 1991; Lankford *et al.*, 1993; Wiertz *et al.*, 1996). Recently, Ubc6p and Ubc7p together with an additional component, Cue1p, have been shown to be necessary for retrograde transport of aberrant ER proteins as well as for their degradation by the proteasome (Hiller *et al.*, 1996; Biederer *et al.*, 1997; Plemper *et al.*, 1997). It is thus possible that signals similar to those implicated in retrograde transport and degradation of ER proteins could also act as degradation signals for proteins located in the cytoplasm.

As particular sequence motifs are shared by some but not by all of the C-terminal appendages, it is possible that several pathways, utilizing the Ubc6p/Ubc7p pair, but recognizing different signals are involved. The previously characterized 67 amino acid residue N-terminal sequence of the Mat α -2 repressor (Deg1) contains a strong signal for degradation involving the Ubc6p and Ubc7p enzymes (Hochstrasser and Varshavsky, 1990; Chen *et al.*, 1993). Surprisingly, the Deg1 signal has little in common, not even hydrophobicity, with C-terminal sequences described here, suggesting that it may be degraded via a different Ubc6p plus Ubc7p subpathway.

In the work described here, we have exploited only part of the potential of libraries producing β -galactosidase or Ura3p C-terminal fusion proteins. These libraries make it possible not only to search systematically for degradation

signals but also, potentially, to find new ubiquitin system target proteins and to identify the branches of the ubiquitin system involved in their degradation. Further, the unstable fusion protein clones can be used as tools to clone novel genes, such as E3 genes, acting downstream of the Ubc enzyme involved in their breakdown.

Materials and methods

Yeast and bacterial media and methods

Yeast were grown in rich (YPD) and minimal (SD) media, prepared as described (Sherman *et al.*, 1986). For induction of *GAL1* promoter-dependent genes in liquid culture, 2% galactose and 2% raffinose were substituted for glucose in SD medium. For induction of the *CUP1* promoter, CuSO₄ was added to a final concentration of 200 μ M 8–12 h before radiolabeling. Standard yeast genetic methods were used (Sherman *et al.*, 1986). *Escherichia coli* strains used were DH5 α and MC1061 (Raleigh *et al.*, 1989). Standard techniques were used for DNA recombinant work (Sambrook *et al.*, 1989). Colonies producing β -galactosidase were detected by plating on minimal medium without glucose containing 115 mM potassium phosphate buffer pH 7.0, 2% raffinose, 2% galactose and 40 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal).

Yeast strains

Table II lists the strains used in the present study. In all degradation studies, yeast strains with *ubc* or other mutations were compared with otherwise isogenic cells carrying the corresponding wild-type gene.

Plasmid and library construction

The pBRR vectors (Figure 1A) were derived from the pLGSD5 plasmid (Bachmair *et al.*, 1986; called G1 in Guarente, 1983). The *Bam*HI site and two of the three *Eco*RI sites of pLGSD5 were eliminated, leaving a single *Eco*RI site in the C-terminal coding region of the *lacZ* gene. Into this *Eco*RI site we inserted an *Eco*RI fragment containing the C-terminal coding region plus the cloning site of one of the *E.coli lacZ* vectors pUR278, pUR288 or pUR289 (Rüther and Müller-Hill, 1983) to yield the yeast *lacZ* expression vectors pBRR78, pBRR88 and pBRR89, respectively. Each of these vectors produces C-terminal β -galactosidase fusions in a different reading frame. To prepare the *lacZ* C-terminal fusion protein library, we inserted yeast genomic *Sau*3AI fragments of 0.5–2 kb into the *Bam*HI site of each of the vectors separately. Before selection, the three libraries were mixed in equal amounts. The library consists of ~100 000 clones.

The plasmid expressing N-terminal HA epitope-tagged β -galactosidase–SL17 fusion protein (HAL–SL17) was made as follows. PB67, a plasmid derived from pLGSD5 with a HA epitope inserted after its N-terminal ATG, was obtained from S.Sadis. A 2.3 kb *Xho*I–*Sac*I restriction fragment encoding the N-terminal part of the HA-tagged *lacZ* region of PB67 was used to replace the corresponding *Xho*I–*Sac*I fragment of the *lacZ*–SL17 fusion protein plasmid.

The pOC9 vector (Figure 1B) was derived from the pRS414 *TRP1* centromeric vector (Sikorski and Hieter, 1989). A *Bam*HI–*Eco*RI fragment containing the *CUP1* promoter from plasmid pYSK12-15 (Ecker *et al.*, 1987) was inserted into pRS414 producing the pOC6 plasmid. The *Bam*HI site of pOC6 was destroyed to yield the pOC7 vector. Into the *Eco*RI site of pOC7 under the control of the *CUP1* promoter we inserted a *URA3* gene fused by PCR to a C-terminal HA epitope followed by a *Bam*HI cloning site and a translation stop signal, to produce the pOC9 vector. To make the Ura3 C-terminal fusion protein library, fragments of 4–9 kb from a partial *Sau*3AI digest of yeast genomic DNA were ligated into the *Bam*HI site of the pOC9 vector. The library consisted of 150 000 clones, one-third of which had inserts detectable by PCR.

Selection of clones producing unstable fusion proteins

The pBRR library was transformed into wild-type yeast (*BWG1-7a*) and plated on X-gal plates. Out of 40 000 colonies, 3500 white colonies were picked. These were presumed to include colonies producing unstable *lacZ* fusion proteins. DNA extracted from the pooled white colonies was transformed into the *pre1-1pre2-1* proteasome subunit mutant (Heinemeyer *et al.*, 1993) and blue colonies were picked and pooled. The DNA from these pooled blue colonies was screened a second time with wild-type followed by *pre1-1pre2-1* mutant cells. The

Table II. Yeast strains

Strain	Genotype	Source
BWG1-7a	<i>MATa ura3;leu2;his4;ade1</i>	Sadis <i>et al.</i> (1995)
WCG4a	<i>MATa ura3;leu2-3,112;his3-11,5</i>	Heinemeyer <i>et al.</i> (1993)
WCG4a-11/21a	<i>MATa ura3;leu2-3,112;his3-11,5 pre1-1pre2-1</i>	Heinemeyer <i>et al.</i> (1993)
yHI29/W	<i>MATa ura3;leu2-3,112;his3-11,5</i>	Hilt <i>et al.</i> (1993)
yHI29/14	<i>MATa ura3;leu2-3,112;his3-11,5 pre1-1pre4-1</i>	Hilt <i>et al.</i> (1993)
SUB62/DF5a	<i>MATa ura3-52; leu2-3; his3Δ200;lys2-801;trp1-1(am)</i>	Finley <i>et al.</i> (1987)
YWO5	<i>MATa ubc1::HIS3</i> (in SUB62)	Seufert <i>et al.</i> (1990)
Y531	<i>MATa rad6 null</i> (in SUB62)	this paper
MHY508	<i>MATa ubc4::HIS3;ubc5::LEU2</i> (in SUB62)	Seufert and Jentsch (1990)
SS410	<i>MATa ubc6::HIS3</i> (in SUB62)	Sommer and Jentsch (1993)
SS411	<i>MATa ubc7::LEU2</i> (in SUB62)	Jungmann <i>et al.</i> (1993)
SS414	<i>MATa ubc6::HIS3;ubc7::LEU2</i> (in SUB62)	Chen <i>et al.</i> (1993)
ubc11	<i>MATa ubc11 null</i> (in SUB62)	from D.Finley
UTL-7A	<i>MATa ura3-52;leu2-3,2-112;his3Δ200;lys2-801;trp1-1(am)</i>	Wiebel and Kunau (1992)
pas2	<i>MATa ubc10::LEU2</i> (in UTL-7A)	Wiebel and Kunau (1992)
KY130	<i>MATa ura3-52;leu2-Δ2,2-112;bas1-1;bas2-2;gcn4-Δ1;ade8-GCN4;GAL</i>	Kornitzer <i>et al.</i> (1994)
KY203	<i>MATa cdc34-2</i> (in KY130)	Kornitzer <i>et al.</i> (1994)
SUB61/DF5α	<i>MATα ura3-52; leu2-3; his3Δ200;lys2-801;trp1-1(am)</i>	Finley <i>et al.</i> (1987)
YWO102	<i>MATα ubc9-Δ1::TRP1;LEU2::ubc9-1</i> (in SUB61)	Seufert <i>et al.</i> (1995)
MHY501	<i>MATα ura3-52; leu2-3,2-112; his3Δ200;lys2-810;trp1-1;cam1 DOA4</i>	Papa and Hochstrasser (1993)
MHY623	<i>MATα ura3-52; leu2-3,2-112;his3Δ200;lys2-810;trp1-1;cam1 doa4</i>	Papa and Hochstrasser (1993)

DNA from pooled blue colonies in the *pre1-1pre2-1* background from the second screen was transformed into wild-type cells and individual white colonies were isolated. The β -galactosidase fusion proteins of these colonies were tested for instability by β -galactosidase assay and by pulse-chase analysis. Three clones producing unstable fusion proteins were isolated. One of these, SL17 (derived from the pBRR88 vector), was stabilized in the *ubc6*, *ubc7* or *ubc6ubc7* null mutants and has been studied in detail in this investigation.

The pOC9 library was transformed into wild-type yeast cells (*SUB62*) and 30 000 colonies growing on SD without tryptophan were replica-plated onto tryptophan-free 5-FOA plates (Sikorski and Boeke, 1991). After incubating for 1 day, 4000 surviving colonies, with zero or low Ura3 activity, were transferred as patches to SD plates without tryptophan. A replica of these patches was made on SD plates lacking uracil, containing 200 μ M CuSO₄ to induce Ura3p synthesis, and growing colonies were isolated. The purpose of this additional selection was to choose clones with low Ura3p activity, which are likely to include those producing unstable fusion proteins, and to eliminate clones with zero Ura3p activity. In this selection, 345 colonies were isolated, pooled and DNA was prepared from them. This DNA was transformed into the *ubc6ubc7* double null mutant and was plated onto SD without tryptophan. Replicas on SD without uracil were made and growing colonies were picked. These were presumed to produce labile fusion proteins stabilized in a *ubc6ubc7* background. The inserts of these plasmids were sequenced, and unique clones were back-transformed into wild-type cells and tested for instability by pulse-chase analysis. C-terminal extensions were sequenced by the dye terminator cycle sequencing reaction and the gel run on a Perkin Elmer, ABI-prism™ 377 DNA sequencer. All C-terminal extension sequences were compared with the *Saccharomyces* Genome Database (SGD).

β -galactosidase assay

The enzymatic activity of β -galactosidase from mid-exponential cultures growing on SD medium without glucose plus 2% galactose and 2% raffinose was measured as described by Guarente (1983).

Pulse-chase analysis

Cells transformed with the plasmids of interest were grown at 30°C in selective medium without methionine to $A_{600} = 0.3$ – 0.8 (mid-exponential phase). The cells were harvested by centrifugation and resuspended at $A_{600} = 6.0$ in the same medium. Cells (1 ml) were labeled for 5 min at 30°C with 200 μ Ci of [³⁵S]L-methionine (Du Pont). The cells were washed three times with chase medium (containing SD medium with 1 mg/ml L-methionine and 0.5 mg/ml cycloheximide). The cells were resuspended in 6 ml of chase medium. Samples of 1.5 ml, withdrawn during the incubation, harvested by centrifugation and suspended in 0.4 ml of IP buffer (150 mM NaCl, 50 mM Tris-HCl buffer pH 7.5, 5 mM EDTA and 1% Triton X-100) supplemented with a mixture of

protease inhibitors [10 mM *N*-ethylmaleimide (NEM), 1 mM phenyl-methylsulfonyl fluoride (PMSF), 2 mg/ml pepstatin A, 10 mg/ml aprotinin, 5 mg/ml phosphoramidon (*N*- α -rhamnopyranosyloxyhydroxy-phosphinyl)-Leu-Trp, 20 mg/ml chymostatin, 5 mg/ml E-64 (*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane)]. An equal volume of 0.5 mm glass beads was added, and the cells were disrupted by vortexing three times for 1 min, with intermittent cooling. The extracts were centrifuged at 15 000 *g* for 15 min. Samples of the supernatants were removed to determine trichloroacetic acid (TCA)-insoluble ³⁵S c.p.m. Samples of the supernatants containing equal amounts of TCA-insoluble ³⁵S c.p.m. were immunoprecipitated with appropriate antibodies, anti- β -galactosidase (Promega) or anti-HA epitope (Babco). The antibody-antigen complexes were adsorbed onto protein A beads (Santa-Cruz), washed three times with IP buffer containing 0.1% SDS, and eluted with SDS-PAGE sample buffer followed by electrophoresis. The SDS-PAGE radioactivity patterns were detected and quantitated with a Fujix Bas1000 phosphorimager.

Acknowledgements

We thank Bella Baumgarten for valuable assistance. Generous gifts of plasmids and yeast strains from Mark Hochstrasser, Stefan Jentsch, Daniel Kornitzer, Seth Sadis and Dieter Wolf are gratefully acknowledged. Special thanks are due to Daniel Finley for hospitality in his laboratory during the early stages of the work and for stimulating discussions. We are grateful to Yael Altuvia for help with sequence analysis. This work was supported by grants from The United States-Israel Binational Science Foundation (BSF), The Israel Science Foundation and by a joint grant from the German and Israeli Science Ministries.

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Received October 1, 1997; revised March 16, 1998;
accepted March 25, 1998