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The N-end rule pathway controls the import of peptides through degradation of a transcriptional repressor

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Ubiquitin-dependent proteolytic systems underlie many processes, including the cell cycle, cell differentiation and responses to stress. One such system is the N-end rule pathway, which targets proteins bearing destabilizing N-terminal residues. Here we report that Ubr1p, the main recognition component of this pathway, regulates peptide import in the yeast *Saccharomyces cerevisiae* through degradation of Cup9p, a 35 kDa homeodomain protein. Cup9p was identified using a screen for mutants that bypass the previously observed requirement for Ubr1p in peptide import. We show that Cup9p is a short-lived protein ($t_{1/2}$ ~5 min) whose degradation requires Ubr1p. Cup9p acts as a repressor of *PTR2*, a gene encoding the transmembrane peptide transporter. In contrast to engineered N-end rule substrates, which are recognized by Ubr1p through their destabilizing N-terminal residues, Cup9p is targeted by Ubr1p through an internal degradation signal. The Ubr1p–Cup9p–Ptr2p circuit is the first example of a physiological process controlled by the N-end rule pathway. An earlier study identified Cup9p as a protein required for an aspect of resistance to copper toxicity in *S.cerevisiae*. Thus, one physiological substrate of the N-end rule pathway functions as both a repressor of peptide import and a regulator of copper homeostasis.

Keywords: *CUP9*/N-end rule/peptide import/proteolysis/ *PTR2*/*UBR1*

Introduction

Many regulatory proteins are short-lived *in vivo* (Schwob *et al.*, 1994; King *et al.*, 1996; Varshavsky, 1996). This metabolic instability makes possible rapid adjustment of the protein's concentration (or subunit composition) through changes in the rates of its synthesis or degradation. Protein degradation plays a role in a multitude of processes, including cell growth, division, differentiation and responses to stress. In eukaryotes, a large fraction of intracellular proteolysis is mediated by the ubiquitin system. Ubiquitin (Ub) is a 76-residue protein whose covalent conjugation to other proteins marks them for processive degradation by the 26S proteasome—an ATP-dependent, multisubunit protease (Jentsch and Schlenker, 1995; Hilt and Wolf, 1996; Hochstrasser, 1996; Rubin *et al.*, 1997).

Features of proteins that confer metabolic instability are called degradation signals (degrons). One of the degradation signals recognized by the ubiquitin system is called the N-degron. It comprises two essential determinants: a destabilizing N-terminal residue and an internal lysine of a substrate (Bachmair *et al.*, 1986; Varshavsky, 1996). The Lys residue is the site of formation of a substrate-linked multiubiquitin chain (Bachmair and Varshavsky, 1989; Chau *et al.*, 1989). A set of N-degrons bearing different N-terminal residues that are destabilizing in a given cell type yields a rule, called the N-end rule, which relates the *in vivo* half-life of a protein to the identity of its N-terminal residue. Similar but distinct versions of the N-end rule operate in all organisms examined, from mammals to fungi and bacteria (Varshavsky, 1996).

The N-end rule pathway is organized hierarchically. In eukaryotes such as *Saccharomyces cerevisiae*, Asn and Gln are tertiary destabilizing N-terminal residues in that they function through their conversion, by the *NTA1*-encoded N-terminal amidase (Nt-amidase), into the secondary destabilizing residues Asp and Glu (Baker and Varshavsky, 1995). Secondary residues, in turn, function through their conjugation to Arg by the *ATE1*-encoded Arg-tRNA–protein transferase (R-transferase) (Balzi *et al.*, 1990). Arg is one of several primary destabilizing N-terminal residues which are bound directly by N-recognin, a 225 kDa E3 protein encoded by the *UBR1* gene (Bartel *et al.*, 1990). Ubr1p, together with the associated ubiquitin-conjugating (E2) enzyme Ubc2p, mediates the formation of a substrate-linked multiubiquitin chain (Varshavsky, 1996).

The N-end rule pathway was first encountered in experiments that explored, in *S.cerevisiae*, the metabolic fate of a fusion between Ub and a reporter such as *Escherichia coli* β-galactosidase (β-gal) (Bachmair *et al.*, 1986). While such engineered N-end rule substrates have been extensively characterized (Varshavsky, 1996), little is known about their physiological counterparts. The few identified so far include the *GPA1*-encoded Gα subunit of the *S.cerevisiae* heterotrimeric G protein, which mediates the pheromone response in this fungus, and RNA polymerases of alphaviruses whose hosts include mammalian and insect cells (de Groot *et al.*, 1991; Madura and Varshavsky, 1994). Physiological functions of the instability of these proteins remain to be understood (Varshavsky, 1996). Inactivation of the N-end rule pathway in *S.cerevisiae*—through deletion of the *UBR1* gene—results in cells which grow slightly slower than their wild-type counterparts, and are impaired in sporulation (increased frequency of asci containing fewer than four spores), but otherwise appear to be normal (Bartel *et al.*, 1990).

Recently, Becker and colleagues (Alagramam *et al.*, 1995) have reported that *ubr1*Δ cells are deficient in the

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A

Strain	Genotype	Peptide import	Strain	Genotype	Peptide import
JD53	wt	+	MHY599	*pas2Δ*	+
JD83-1A	*ubr1Δ*	–	CBY8	*ubc2Δ, ubc1Δ*	+
KM207-1	*ate1Δ*	+	CBY11	*ubc2Δ, cdc34-1*[1]	+
SGY6	*nta1Δ*	+	EJY105/6	*ubc2Δ, ubc4Δ*	–
CBY20	*ptr2Δ*	+	CBY4	*ubc2Δ, ubc5Δ*	+
EJ2	*ubc1Δ*	+	CBY5	*ubc2Δ, ubc6Δ*	+
BBY67	*ubc2Δ*	+/–	CBY6	*ubc2Δ, ubc7Δ*	+
JD34	*ubc2Δ*	+/–	CBY7	*ubc2Δ, ubc8Δ*	+
EJY102	*ubc2Δ*	+/–	CBY9	*ubc2Δ, ubc9-1*[1]	+
RJD549	*cdc34-1*[1]	+	CBY10	*ubc2Δ, pas2Δ*	+
RJD795	*cdc34-2*[1]	+	EJ10	*ubc4Δ, ubc5Δ*	+
EJ3	*ubc4Δ*	+	MHY503	*ubc4Δ, ubc6Δ*	+
EJ1	*ubc5Δ*	+	MHY554	*ubc4Δ, ubc7Δ*	+
MHY495	*ubc6Δ*	+	MHY552	*ubc6Δ, ubc7Δ*	+
MHY507	*ubc7Δ*	+	MHY550	*ubc4Δ, 5Δ, 6Δ*	+
MHY601	*ubc8Δ*	+	MHY557	*ubc4Δ, 6Δ, 7Δ*	+
YWO55	*ubc9-1*[1]	+	MHY570	*ubc4Δ, 5Δ, 6Δ, 7Δ*	+

Fig. 1. The import of peptides is decreased in the absence of Ubc2p and virtually abolished in the absence of Ubc2p and Ubc4p. *Saccharomyces cerevisiae* mutants deficient in one or more ubiquitin-conjugating (E2) enzymes or other components of the ubiquitin system were tested for their ability to import peptides, using the halo assay and a toxic dipeptide L-leucyl-L-ethionine (Leu-Eth) (see Materials and methods). '+', '±' and '-' denote, respectively, the apparently wild-type, significantly reduced, and undetectable levels of peptide import. A superscript '1' refers to strains that carried a *ts* allele of an essential E2 enzyme, either Cdc34p (Ubc3p) or Ubc9p; the 30°C temperature of the test was semi-permissive for these strains. (**A**) Summary of the results. (B–F) Examples of the actual halo assays, with wild-type (**B**), *ptr2*Δ (**C**), *ubr1*Δ (**D**), *ubc2*Δ (**E**), *ubc4*Δ (**F**) and *ubc2*Δ *ubc4*Δ (**G**) strains of *S.cerevisiae*. Elimination of some E2 enzymes in the *ubc2*Δ background restored halo formation, presumably because such strains were growth-impaired in a way that made them hypersensitive to the toxicity of ethionine. By contrast, although *ubc2*Δ *ubc4*Δ cells were also growth-impaired, they were import-defective and therefore grew in the immediate vicinity of the filter.

import of di- and tripeptides, suggesting that this process, which is universal among living cells, requires the N-end rule pathway. In the present work, we identified the underlying regulatory mechanism and discovered a new physiological substrate of the N-end rule pathway, the homeodomain protein Cup9p. This short-lived protein is targeted for degradation by Ubr1p, and acts as a transcriptional repressor of *PTR2*, a gene that encodes a transmembrane peptide transporter. The Ubr1p–Cup9p–Ptr2p circuit is the first example of a physiological process controlled by the N-end rule pathway.

Results

The involvement of Ubc2p and Ubc4p E2 enzymes in the control of peptide import

We began by asking whether components of the N-end rule pathway other than Ubr1p were also necessary for the import of peptides. Previous work has shown that Ubc2p, one of 13 ubiquitin-conjugating (E2) enzymes of *S.cerevisiae*, is required for the degradation of engineered N-end rule substrates, and is physically associated with the E3 protein Ubr1p (N-recognin) (Jentsch, 1992; Madura *et al.*, 1993).

To test for the ability of *S.cerevisiae* to import peptides, we used a halo assay, in which a filter soaked in the toxic dipeptide L-leucyl-L-ethionine (Leu-Eth) is placed on a plate, inhibiting the growth of import-competent cells near the filter. By this test, the elimination of *UBC2* impaired, but did not abolish, the import of peptides (Figure 1A, B and E). To determine which of the other E2 enzymes, if any, were required for the residual peptide import observed in *ubc2*Δ cells, a number of single and multiple mutants in *UBC* genes were examined (Figure 1). We found that the elimination of both *UBC2* and *UBC4* virtually abolished the import of peptides (Figure 1A, B and E–G). Elimination of Ubc4p, one of the more abundant E2 enzymes (Bachmair *et al.*, 1986; Jentsch, 1992), had previously been noticed to decrease slightly the activity of the N-end rule pathway (Bartel, 1990). Cells lacking either Nta1p or Ate1p—the 'upstream' components of this pathway—were also examined and found unimpaired in the import of peptides, in contrast to *ubr1*Δ and *ubc2*Δ *ubc4*Δ cells (Figure 1A).

Identification of Cup9p as a negative regulator of peptide import

Previous work (Alagramam *et al.*, 1995) has shown that deletion of *UBR1* greatly reduces the level of *PTR2* mRNA, which encodes the transmembrane peptide transporter. This result, and our observation that peptide import requires the presence of at least one of two specific ubiquitin-conjugating enzymes (Figure 1), suggested a model in which expression of the Ptr2p transporter is regulated by a short-lived repressor that is degraded by the N-end rule pathway. In cells lacking *UBR1*, the repressor would be expected to accumulate, thereby blocking peptide import. One prediction of this model is that inactivation of this repressor would bypass the requirement for Ubr1p in peptide import. A screen for such 'bypass' mutations (see Materials and methods) yielded 199 recessive isolates, of which 101 defined one complementation group, termed

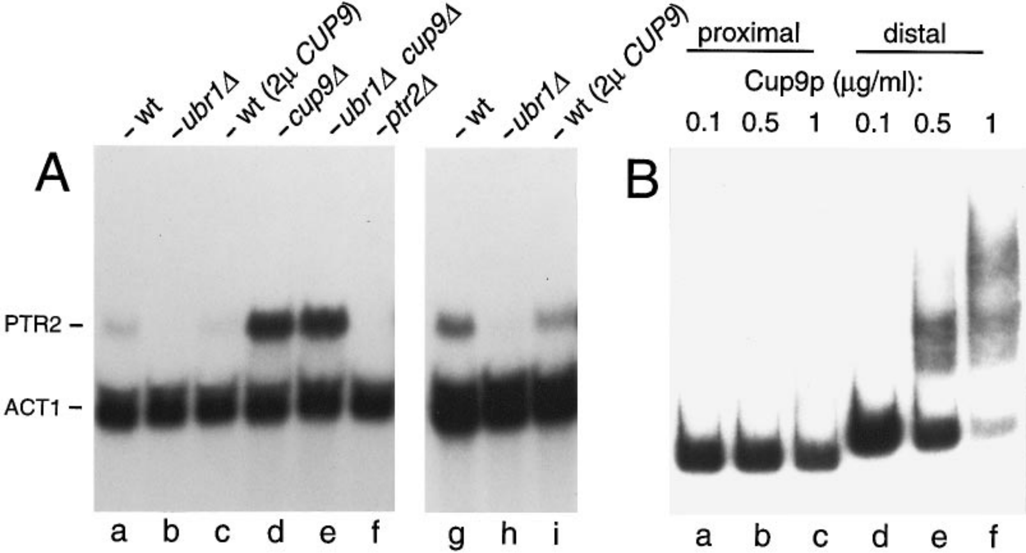

Fig. 2. Cup9p is a repressor of the *PTR2* gene. (**A**) Expression of the *S.cerevisiae PTR2* gene in different genetic backgrounds. Equal amounts of total RNA isolated from different strains were analyzed by Northern hybridization (see Materials and methods), using the *PTR2* (peptide transporter) and *ACT1* (actin) genes as ^{32}P-labeled probes. Lane a, JD52 (*CUP9 UBR1*) (wild-type) transformed with pCB201 (empty vector). Lane b, JD55 (*CUP9 ubr1*Δ) transformed with pCB201. Lane c, JD52 (*CUP9 UBR1*) transformed with the high-copy plasmid pCB209 that expressed *CUP9* from its natural promoter. Lane d, CBY18 (*cup9*Δ *UBR1*). Lane e, CBY16 (*cup9*Δ *ubr1*Δ). Lane f, CBY21 (*CUP9 UBR1 ptr2*Δ). Lanes g–i, same as lanes a–c, but a longer autoradiographic exposure to highlight the tight repression of *PTR2* by Cup9p in *ubr1*Δ cells (b, h) and the difference between levels of *PTR2* mRNA in the wild-type cells (a, g) and their counterparts that overexpressed Cup9p (c, i). (**B**) Cup9p specifically binds to a site in the P_{PTR2} promoter. A gel shift assay with Cup9-H_6, poly-dI·dC and ^{32}P-labeled DNA fragments of the *PTR2* promoter hybridization (see Materials and methods). Lanes a–c, with a fragment (–1 to –447) proximal to the inferred start codon of the *PTR2* ORF. Lanes d–f, same as lanes a–c, but with a more distal DNA fragment (–448 to –897). Concentrations of Cup9-H_6 (in μg/ml) are indicated above the lanes.

sub1 (suppressor of a block to peptide import in *ubr1*Δ). In agreement with the model's prediction, *sub1* mutants acquired the ability to express *PTR2* in the absence of *UBR1* (Figure 2A, lane b versus lane e). The *sub1* locus was cloned by complementation (see Materials and methods), and was found to be the *CUP9* gene.

CUP9 was originally identified by Knight *et al.* (1994) as a gene whose disruption impairs the copper resistance of *S.cerevisiae* growing on lactate, a non-fermentable carbon source. Under these conditions, Cup9p plays a major (but mechanistically obscure) role in copper homeostasis (Knight *et al.*, 1994). *CUP9* encodes a 35 kDa protein that contains a homeodomain, an ~60-residue helix–turn–helix DNA-binding motif present in many eukaryotic regulatory proteins (Wolberger, 1996). Outside the homeodomain region, the sequence of Cup9p is not similar to sequences in databases.

To verify that *CUP9* and *SUB1* were the same gene, complementation tests were carried out. Two independently derived *ubr1*Δ *cup9::LEU2* strains (CBY16 and CBY17) were crossed to *ubr1*Δ *sub1-1* (CBY15), and the resulting diploids (CBY23 and CBY24, respectively) were tested for their ability to import dipeptides (see Materials and methods). As would be expected of allelic loci, *cup9::LEU2* and *sub1-1* failed to complement one another: both diploid strains remained import-competent (data not shown). In another test, CBY23 and CBY24 were sporulated, and the segregants were analyzed for the presence of the *LEU2* gene (integrated at the *CUP9* locus) and for the ability to import peptides. Among the eight tetrads tested, *LEU2* was present in two of the four segregants, whereas all four segregants were invariably import-competent, a pattern expected if *CUP9* and *SUB1* were one and the same gene.

To examine the regulation of peptide import by Cup9p and Ubr1p, congenic *S.cerevisiae* strains that lacked, expressed or overexpressed Cup9p and/or Ubr1p were constructed and assayed for peptide import by growth on selective media. Suspensions of cells (auxotrophic for lysine) were serially diluted and plated on either rich media, minimal media lacking lysine and containing Lys-Ala dipeptide (selecting for peptide import), or minimal media containing the toxic dipeptide Leu-Eth (selecting against peptide import). All strains grew at comparable rates on rich media (Figure 3A). On minimal media that supplied the essential lysine as the Lys-Ala dipeptide, the *CUP9 UBR1* (wild-type), *cup9*Δ *UBR1* and *cup9*Δ *ubr1*Δ strains grew at comparable rates (Figure 3B), whereas the *CUP9 ubr1*Δ strain failed to grow (Figure 3B), in agreement with the observation that *UBR1* is required for peptide import (Figure 1). Opposite growth patterns were observed on media containing toxic dipeptide (Figure 3C). Comparison of the data in Figure 3 with *PTR2* mRNA levels (Figure 2A) suggested that *CUP9* exerts its effect on the import of peptides by repressing transcription of *PTR2*. For example, wild-type (*CUP9 UBR1*) cells overexpressing Cup9p exhibited reduced levels of *PTR2* mRNA (Figure 2A) and decreased sensitivity to toxic dipeptide (Figure 3C), whereas strains lacking *CUP9* (*cup9*Δ *UBR1* and *cup9*Δ *ubr1*Δ) overexpressed *PTR2* (Figure 2A) and were hypersensitive to toxic dipeptide (Figure 3C).

Cup9p is a repressor of the PTR2 gene

To address the mechanism of repression by Cup9p, we asked whether purified Cup9p (see Materials and methods) could selectively bind to specific regions of the *PTR2* promoter. Gel shift assays in the presence of poly-dI·dC competitor DNA were performed with labeled DNA

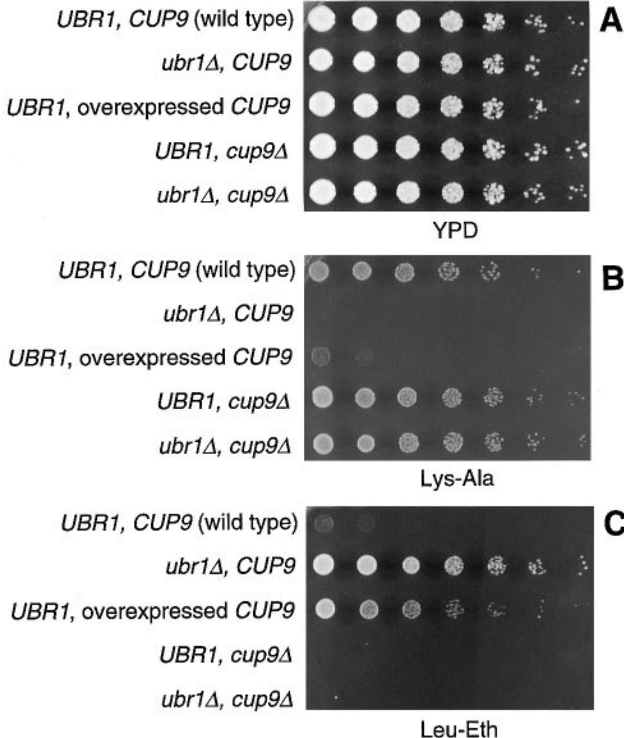

Fig. 3. Relative capacity for peptide import in congenic *S.cerevisiae* strains that contained, lacked or overexpressed Cup9p and/or Ubr1p. Serial dilutions of the indicated strains were deposited by a 48-pin applicator onto either rich (YPD) medium (**A**), minimal medium containing 66 μM Lys-Ala dipeptide as the sole source of lysine (**B**), or minimal medium containing both lysine (at 110 μM) and the toxic dipeptide Leu-Eth (at 55 μM) (**C**). The plates were incubated at 30°C for 1–2 days. The relevant genetic loci are shown on the left. The '*UBR1*, overexpressed *CUP9*' strain (JD52-2μ-CUP9) carried the high-copy plasmid pCB209 that expressed Cup9p from its natural promoter.

fragments of the *PTR2* promoter and His_6-tagged Cup9p (Cup9p-H_6) purified from *E.coli*. Cup9p-H_6 bound to a site within a distal region of the *PTR2* promoter (positions −448 to −897 relative to the inferred *CUP9* start codon), but did not bind to the proximal region of the *PTR2* promoter (positions −1 to −447) under the same conditions (Figure 2B).

The transcriptional repressor function of Cup9p was further suggested by the finding that the co-repressor complex Tup1p/Ssn6p plays a role in the control of peptide import. The Tup1p/Ssn6p complex inhibits transcription of many yeast genes through interactions with gene-specific DNA-binding repressors such as Matα2p (Chen *et al.*, 1993; Smith *et al.*, 1995), a homeodomain homolog of Cup9p. We found that most of our *sub* mutants that were not *CUP9* mutants could be complemented by low-copy plasmids bearing *SSN6* or *TUP1* (G.Turner, S.Saha and A.Varshavsky, unpublished data). In addition, deletion of *SSN6*, like deletion of *CUP9*, restored the ability of *ubr1*Δ cells to import peptides (data not shown).

Ubr1p-dependent degradation of Cup9p

The fact that deletion of *CUP9* renders *PTR*2 transcription independent of Ubr1p (Figure 2A), and the observation that overexpression of Ubr1p enhances the import of peptides in Cup9p-expressing strains (data not shown) suggested that the N-end rule pathway regulates peptide import by targeting Cup9p for degradation. To test this conjecture, we carried out pulse–chase experiments with a C-terminally FLAG-tagged Cup9p in *UBR1* and *ubr1*Δ cells. Cup9p-FLAG was a very short-lived protein ($t_{1/2}$ ~5 min) in *UBR1* cells (Figure 4). By contrast, Cup9p was much longer-lived ($t_{1/2}$ >30 min) in *ubr1*Δ cells (Figure 4). Degradation of Cup9p was also found to depend upon *UBC2* and *UBC4* (data not shown), in agreement with the observation that a *ubc2*Δ *ubc4*Δ double mutant failed to import dipeptides (Figure 1G). The residual instability of Cup9p in *ubr1*Δ cells (Figure 4) suggested the presence of a second, Ubr1p-independent degron; this pattern is reminiscent of another homeodomain repressor, Matα2p, which also contains at least two distinct degradation signals (Hochstrasser and Varshavsky, 1990; Chen *et al.*, 1993).

Ubr1p recognizes engineered N-end rule substrates through their destabilizing N-terminal residues (Varshavsky, 1996). To determine the N-terminal residue of Cup9p, we overexpressed and purified Cup9p-FLAG from *ubr1*Δ *S.cerevisiae* and subjected it to N-terminal sequencing. Cup9p-FLAG was found to have a blocked (presumably acetylated) N-terminus (see Materials and methods), suggesting that Ubr1p targets Cup9p through a degron distinct from the N-degron. Independent evidence for this conjecture was produced through the analysis of GST-Cup9p-ha_2, a fusion of glutathione transferase (GST) and C-terminally ha-tagged Cup9p. Pulse–chase analysis of GST-Cup9p-ha_2 revealed that the fusion protein was nearly as short-lived *in vivo* as the N-terminally unmodified Cup9p-FLAG (Figure 4, lanes b–e; compare with Figure 5, lanes b–e). If Cup9p were targeted for processive degradation through a destabilizing N-terminal residue, a preliminary proteolytic cleavage(s) of Cup9p would be required to expose such a residue (Varshavsky, 1996). In the case of GST-Cup9p-ha_2, this cleavage would generate a proteolytic fragment consisting of GST and an N-terminal portion of Cup9p preceding the cleavage site. Since free GST has been found to be long-lived when expressed in yeast (data not shown), accumulation of such a proteolytic fragment would be expected to accompany the degradation of GST-Cup9p-ha_2.

Pulse–chase analysis of GST-Cup9p-ha_2, using glutathione–agarose beads to isolate GST-containing proteins (see Materials and methods), indicated that the degradation of GST-Cup9p-ha_2 by the N-end rule pathway was not accompanied by the appearance of a fragment containing the N-terminal GST moiety (Figure 5). This finding strongly suggested that Cup9p bears an 'internal' degron recognized by Ubr1p. Additional support for this conjecture was provided by truncation analysis of a Cup9p–DHFR–myc fusion protein. These experiments indicated that the N-terminal 81 residues of the 306-residue Cup9p are dispensable for its Ubr1p-dependent degradation, strongly suggesting that the relevant degradation signal resides in the C-terminal two-thirds of Cup9p (C.Byrd, I.Davydov and A.Varshavsky, unpublished data). Although these results are fully consistent with the presence of an internal Ubr1p-dependent degron in Cup9p, there remains the less parsimonious possibility that Cup9p is degraded via *trans*-targeting (Johnson *et al.*, 1990). In this process, the two determinants of the N-degron (a destabilizing N-terminal residue and a ubiquitin-accepting internal Lys

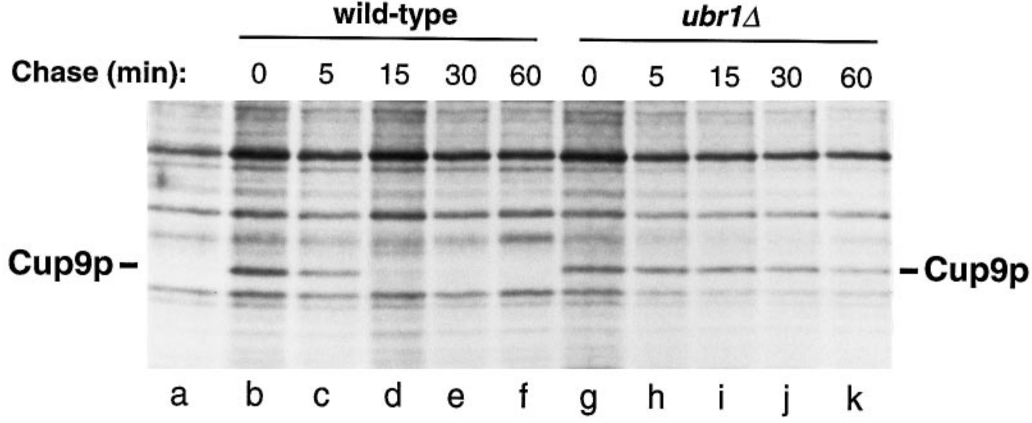

Fig. 4. Degradation of Cup9p by the N-end rule pathway. *Saccharomyces cerevisiae* strains carrying pCB210, a low-copy plasmid that expressed Cup9p-FLAG from the P_{CUP9} promoter, were labeled at 30°C with [^{35}S]methionine/cysteine for 5 min, followed by a chase for 0, 5, 15, 30 and 60 min, preparation of extracts, immunoprecipitation of Cup9p-FLAG with a monoclonal anti-FLAG antibody, SDS–PAGE and autoradiography/quantitation (see Materials and methods). Lane a, JD55 (*ubr1*Δ) cells that carried pCB200 (vector alone). Lanes b–f, JD52 (*UBR1*) (wild-type) cells that carried pCB210, expressing Cup9p-FLAG. Lanes g–k, same as lanes b–f, but with JD55 (*ubr1*Δ) cells.

residue) reside in two different polypeptides, whose interaction yields an active N-degron, leading to ubiquitinylation and degradation of the polypeptide bearing the Lys residue (Johnson *et al.*, 1990). At present, little is known about the protein ligands of Cup9p, save for the likely possibility that Cup9p interacts with the Tup1p/Ssn6p repressor complex (see above), similarly to the previously established interaction of this complex with Matα2, a homeodomain-containing homolog of Cup9p (Smith *et al.*, 1995).

Discussion

The discovery that Ubr1p controls the import of peptides through degradation of the Cup9p repressor (Figure 6) identifies the first clear physiological function of the N-end rule pathway. The existence of mammalian, plant and bacterial homologs of the yeast Ptr2p transporter suggests that the import of peptides in these organisms may also be regulated by the N-end rule pathway.

Why was this pathway, rather than another Ub-dependent proteolytic system, recruited in the course of evolution for the regulation of peptide import? A plausible explanation is suggested by the ability of Ubr1p to bind peptides bearing destabilizing N-terminal residues (Varshavsky, 1996). Since more than half of the 20 amino acids are destabilizing in the yeast and mammalian N-end rules (Varshavsky, 1996), a significant fraction of imported peptides would be expected to compete with Cup9p for binding to Ubr1p. This competition may decrease the rate of Cup9p degradation. [Dipeptides added to a culture of *S.cerevisiae* have been shown to inhibit the N-end rule pathway (Baker and Varshavsky, 1991).] The ensuing increase in the level of Cup9p repressor would in turn decrease the production of the Ptr2p transporter, diminishing the rate of peptide import. This 'peptide-sensing' negative feedback loop would maintain the intracellular concentration of short peptides within a predetermined range—potentially a useful feature, made possible by the substrate-binding properties of Ubr1p. Experiments to verify this model are under way.

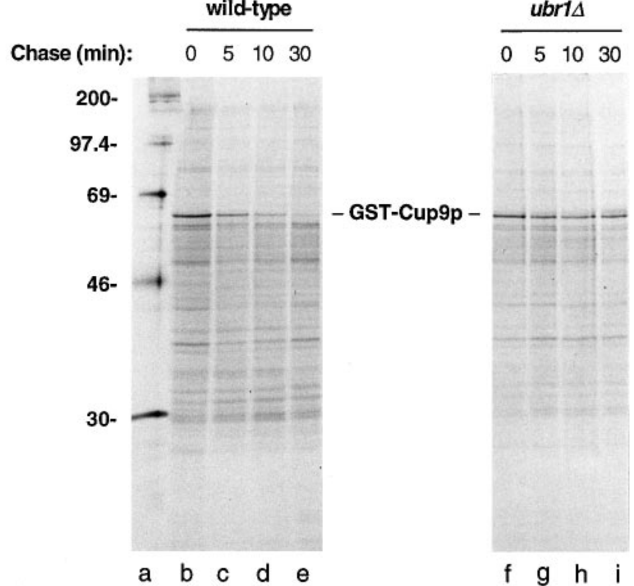

Fig. 5. Degradation of GST-Cup9p-ha_2 is not preceded by a processing that releases a GST-containing fragment. *Saccharomyces cerevisiae* strains carrying pCB120, a low-copy plasmid that expressed GST-Cup9p-ha_2 from the P_{GAL1} promoter, were labeled at 30°C with [^{35}S]methionine/cysteine for 5 min, followed by a chase for 0, 5, 10 and 30 min, preparation of extracts, isolation of GST-Cup9p-ha_2 on glutathione–agarose beads, SDS–PAGE and autoradiography/quantitation (see Materials and methods). Lanes b–e, JD52 (*UBR1*) (wild-type) cells expressing GST-Cup9p-ha_2. Lanes f–i, same as lanes b–e, but with JD55 (*ubr1*Δ) cells. Lane a, molecular mass markers (in kDa).

Another interesting aspect of the Ubr1p–Cup9p–Ptr2p regulatory circuit is its possible relevance to copper homeostasis. In addition to functioning as a repressor of peptide transport, Cup9p also contributes to the resistance of *S.cerevisiae* to copper toxicity during growth on lactate, a non-fermentable carbon source (Knight *et al.*, 1994). Copper homeostasis is mediated by a complex set of circuits, some of which also regulate iron metabolism (Zhou and Thiele, 1993; Ooi *et al.*, 1996). The double role of Cup9p in regulating both peptide import and copper homeostasis may signify a physiological connection between these seemingly unrelated processes.

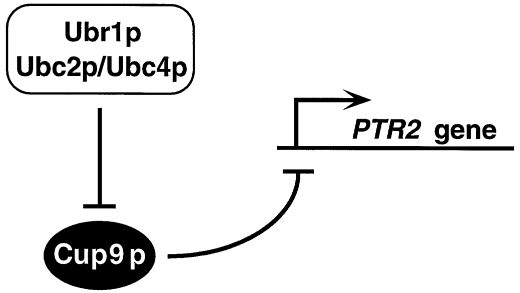

Fig. 6. A model for regulation of peptide import in *S.cerevisiae*. The expression of *PTR2*, which encodes a transmembrane peptide transporter, is regulated by the short-lived, homeodomain-containing transcriptional repressor Cup9p. The concentration of Cup9p is controlled in part through its degradation by the N-end rule pathway, whose targeting components include Ubr1p and either Ubc2p or Ubc4p (see the main text).

Our work adds Cup9p to the list of short-lived regulatory proteins whose degradation is mediated by the ubiquitin system. Many of these proteins are negative regulators. For example, Matα2p, a homeodomain-containing homolog of Cup9p, controls the mating type of *S.cerevisiae* (**a** or α) through repression of **a**-specific genes (Herskowitz, 1989). Matα2p appears to be constitutively short-lived in haploid cells (Hochstrasser and Varshavsky, 1990). Therefore, cessation of Matα2p synthesis upon the conversion of an α cell into an **a** cell results in rapid disappearance of Matα2p and the establishment of **a**-specific circuits (Hochstrassser, 1996). Progression of the cell cycle is also controlled by short-lived negative regulators, in particular by Sic1p, an inhibitor of CDK, the cyclin-dependent kinase (Schwob *et al.*, 1994). In this case, however, a rapid 'on-switch' is based on the phosphorylation-induced degradation of the previously stable Sic1p (King *et al.*, 1996). Whether the Cup9p repressor (Figure 6) is constitutively short-lived or whether its stability is regulated by external conditions such as, for example, different nitrogen sources, remains to be determined.

The finding that Cup9p lacks a destabilizing N-terminal residue indicates that Ubr1p, the recognition component of the N-end rule pathway, is able to target substrates bearing either internal degradation signals or N-degrons. The Gα subunit of the G protein—the other known physiological substrate of Ubr1p in *S.cerevisiae*—also lacks a destabilizing N-terminal residue (Madura and Varshavsky, 1994). Thus, a substantial fraction of naturally short-lived proteins targeted by the N-end rule pathway may bear internal degrons rather than N-degrons, a possibility that the identification of other physiological substrates of this pathway will address.

Materials and methods

Strains, media and genetic techniques

Saccharomyces cerevisiae strains were grown in rich (YPD) medium containing 2% peptone, 1% yeast extract and 2% glucose or in synthetic yeast media, containing 0.67% yeast nitrogen base without amino acids (Difco), auxotrophic nutrients at concentrations specified by Sherman *et al.* (1986) and either 2% glucose (SD medium), 2% raffinose (SM–raffinose medium) or 2% galactose (SM–galactose medium) as carbon sources. SHM–glucose medium used in halo assays was prepared according to Island *et al.* (1987) and was identical to SD medium except that it lacked methionine and contained allantoin (1 mg/ml) and yeast nitrogen base (Difco, 1.7 mg/ml) without amino acids and ammonium sulfate. *Escherichia coli* strain DH5α was used for plasmid propagation and cloning steps. For induction of the P_{GAL1} promoter, cells were grown to an A_{600} of 0.5–1 in SM–raffinose medium, pelleted and transferred to SM–galactose medium for 3 h.

Halo and dilution assays

Peptide import was assayed using the halo and dilution methods. For halo assays (Island *et al.*, 1987), cells were grown to an A_{600} of ~1 in SHM–glucose medium with auxotrophic supplements. Cells were pelleted by centrifugation and resuspended in water to ~5×10^7 cells/ml. Then 0.1 ml of cell suspension was mixed with 10 ml of 0.8% noble agar (Difco) at 55°C, and spread on plates containing 20 ml of SHM medium. Sterile filter disks containing the toxic dipeptide L-leucyl-L-ethionine (Leu-Eth; 5 μmol) were placed in the middle of each plate, followed by incubation at 30°C for 1–2 days.

For dilution assays, strains were grown (under selection for plasmids) in SD medium to an A_{600} of ~1. Cells from each sample (1.5×10^7) were spun down and resuspended in 1 ml of water. The samples were serially diluted by 4-fold in a microtiter plate (150 μl/well) to generate eight different initial concentrations of cells that ranged from 1.5×10^7 to 915 cells/ml. The suspensions were spotted to various media, using a 48-pin applicator. The plates were incubated at 30°C for 1–2 days.

Leu-Eth was synthesized using standard methods of organic chemistry. Briefly, L-ethionine methyl ester (Eth-OMe) was produced from L-ethionine and methanol. Eth-OMe was then coupled with *N-tert*-butoxy-carbonyl-L-leucine-*p*-nitrophenyl ester (N-*t*-BOC-L-leucine-PNP), yielding N-*t*-BOC-L-leucyl-L-ethionine methyl ester, which was purified by flash chromatography on a silica column. N-*t*-BOC-L-leucyl-L-ethionine methyl ester was then converted into N-*t*-BOC-L-leucyl-L-ethionine by treatment with KOH. N-*t*-BOC-L-leucyl-L-ethionine was deprotected with trifluoroacetic acid, yielding Leu-Eth.

A screen for import-competent mutants in the ubr1Δ background

Thirty 5-ml cultures of JD83-1A (*leu2-3 ubr1*Δ), auxotrophic for leucine, were grown to an A_{600} of ~1, and ~1.5×10^7 cells from each culture were plated onto SD medium lacking leucine and histidine but containing the leucyl-histidine dipeptide at 0.23 μM. Plates were incubated at 30°C for 2 days, selecting for mutants able to grow on this medium. Of the 320 sub mutants ('suppressors of a block to peptide import in *ubr1*Δ') thus obtained, 199 were found by complementation tests to be clearly recessive. Among these, 101 mutants belonged to one complementation group, termed *sub1*.

Isolation of the CUP9 (SUB1) gene

A 50 ml culture of *S.cerevisiae* CBY15 (*ubr1*Δ *sub1-1*) was grown to an A_{600} of ~1 in SHM–glucose. Cells were made competent with lithium acetate (Ausubel *et al.*, 1992), and 25 0.1-ml samples (~7×10^7 cells/ml) were transformed with a yeast genomic DNA library (American Type Culture Collection; #77164) in the *TRP1*, *CEN6*-based vector pRS200 (Sikorski and Hieter, 1989). The cells were pelleted, and each sample was resuspended in 1.5 ml of SHM–glucose and incubated at 30°C for 3 h. The cells were pelleted again, each sample was resuspended in 0.1 ml of water, added to 10 ml of 0.8% Noble agar at 55°C, and spread onto SHM–glucose plates lacking Trp and containing Leu-Eth at 37 μM. Of the ~2.5×10^4 Trp^+ transformants plated, 14 could grow in the presence of Leu-Eth. Of these, two yielded the library-derived plasmids, pSUB1-1 and pSUB1-6, that complemented the *sub1* mutation of CBY15. The insert of pSUB1-6 contained the insert of pSUB1-1. Partial sequencing of the ~8.2 kb insert of pSUB1-1 identified it as a region of the *S.cerevisiae* chromosome XVI. Deletion analysis (not shown) localized the complementing activity to an ~2.8 kb *Hin*dIII–*Kpn*I fragment, whose sequence revealed the presence of two ORFs (*CUP9* and SCYPL178W). Further deletion analysis (not shown) localized the complementing activity to the previously isolated (Knight *et al.*, 1994) *CUP9* gene. Verification that *CUP9* and *SUB1* were one and there same gene was carried out as described in Results.

Construction of null cup9 mutants

The ~2.8 kb *Hin*dIII–*Kpn*I genomic DNA fragment containing *CUP9* was ligated to *Hin*dIII–*Kpn*I-cut pRS306ΔSpe1, yielding pCB117. pRS306ΔSpe1 was derived from the *URA3*-bearing pRS306 (Sikorski and Hieter, 1989) through elimination of its *Spe*I site. The *CUP9* ORF was disrupted by inserting an ~2 kb, blunt-ended, *LEU2*-containing *Sal*I fragment from pJJ283 into the *Spe*I site of *CUP9*, yielding pCB119, in which the *LEU2* and *CUP9* ORFs were oriented in opposite directions. An ~5 kb *Hin*dIII–*Pvu*II fragment of pCB119 containing the *cup9::LEU2*

Table I. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	References
DF5	*MAT***a**/*MATα trp1-1/trp1-1 ura3-52/ura3-52 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 lys2-801/lys2-801 gal/gal*	Finley *et al*. (1987)
EJ1	*MATα ubc5Δ::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Derivative of DF5[a]
EJ2	*MATα ubc1Δ::URA3 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Derivative of DF5[a]
EJ3	*MATα ubc4Δ::HIS3 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Derivative of DF5[a]
EJ10	*MATα ubc4Δ::HIS3 ubc5Δ::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Derivative of DF5[a]
EJY102	*MATα ubc2Δ::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Derivative of DF5[a]
EJY105	*MAT***a** *ubc2Δ::LEU2 ubc4Δ::HIS3 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Derivative of DF5[a]
EJY106	*MATα ubc2Δ::LEU2 ubc4Δ::HIS3 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Derivative of DF5[a]
MHY495	*MATα ubc6-Δ1::HIS3 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of DF5[b]
MHY503	*MATα ubc4-Δ1::HIS3 ubc6-Δ1::HIS3 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of DF5[b]
MHY507	*MATα ubc7::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of DF5[b]
MHY550	*MATα ubc4-Δ2::TRP1 ubc5-Δ1::LEU2 ubc6-Δ1::HIS3 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of DF5[b]
MHY552	*MATα ubc6-Δ1::HIS3 ubc7::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of DF5[b]
MHY554	*MATα ubc4-Δ1::HIS3 ubc7::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of DF5[b]
MHY557	MATα ubc4-Δ1::HIS3 ubc6-Δ1::HIS3 ubc7::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801	Derivative of DF5[b]
MHY570	*MATα ubc4-Δ2::TRP1 ubc5-Δ1::LEU2 ubc6-Δ1::HIS3 ubc7::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of DF5[b]
MHY599	*MATα pas2 trp ura3-52 ade1 leu2-3*	Chen *et al*. (1993)
MHY601	*MATα ubc8::URA3 trp1-1 ura3-1 ade2-1 his3-11 leu2-3,112 can1-100*	Chen *et al*. (1993)
YWO55	*MATα ubc9-1 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of DF5[c]
YPH500	*MATα trp1-Δ63 ura3-52 ade2-101 his3-Δ200 leu2-Δ1 lys2-801*	Sikorski and Hieter (1989)
BBY67	*MATα ubc2Δ::LEU2 trp1-Δ63 ura3-52 ade2-101 his3-Δ200 leu2-Δ1 lys2-801*	Derivative of YPH500[d]
KM207-1	*MATα ate1Δ::TRP1 trp1-Δ63 ura3-52 ade2-101 his3-Δ200 leu2-Δ1 lys2-801*	Derivative of YPH500[e]
JD34	*MAT***a** *ubc2Δ::URA3 trp1-Δ63 ura3-52 ade2-101 his3-Δ200 leu2-Δ1 lys2-801*	Derivative of YPH500[f]
JD51	*MAT***a**/*MATα trp1-Δ63/trp1-Δ63 ura3-52/ura3-52 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 lys2-801/lys2-801*	Dohmen *et al*. (1995)
JD52	*MAT***a** *trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Johnson *et al*. (1995)
JD53	*MATα trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Dohmen *et al*. (1995)
JD55	*MAT***a** *ubr1Δ::HIS3 trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Madura and Varshavsky (1994)
JD83-1A	*MATα ubr1Δ::HIS3 trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of JD51[f]
RJD549	*MAT***a** *cdc34-1 trp1 ura3-52 leu2-3*	R.Deshaies[g]
RJD795	*MAT***a** *cdc34-2 trp1 ura3-52 leu2-3*	R.Deshaies[g]
SGY6	*MATα nta1Δ::TRP1 trp1-Δ63 ura3-52 ade2-101 his3-Δ200 leu2-3,112 lys2-801*	S.Grigoryev[h]
CBY4	*MATα ubc2Δ::URA3 ubc5Δ::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Derivative of EJ1
CBY5	*MATα ubc2Δ::LEU2 ubc6-Δ1::HIS3 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of MHY495
CBY6	*MATα ubc2Δ::URA3 ubc7::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of MHY507
CBY7	*MATα ubc2Δ::LEU2 ubc8::URA3 trp1-1 ura3-1 ade2-1 his3-11 leu2-3,112 can1-100*	Derivative of MHY601
CBY8	*MATα ubc2Δ::LEU2 ubc1Δ::URA3 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Derivative of EJ2
CBY9	*MATα ubc2Δ::LEU2 ubc9-1 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of YWO55
CBY10	*MATα ubc2Δ::LEU2 pas2 trp ura3-52 ade1 leu2-3*	Derivative of MHY599
CBY11	*MAT***a** *ubc2Δ::LEU2 cdc34-1 trp1 ura3-52 leu2-3*	Derivative of RJD549
CBY15	*MATα sub1-1 ubr1Δ::HIS3 trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of JD83-1A
CBY16	*MAT***a** *cup9::LEU2 ubr1Δ::HIS3 trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of JD55
CBY17	*MAT***a** *cup9::LEU2 ubr1Δ::HIS3 trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of JD55
CBY19	*MAT***a** *cup9::LEU2 trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of JD52
CBY23	*MAT***a**/*MATα sub1-1/cup9Δ::LEU2 ubr1Δ::HIS3/ubr1Δ::HIS3 trp1-Δ63/trp1-Δ63 ura3-52/ura3-52 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 lys2-801/lys2-801*	Produced by mating CBY15 and CBY16
CBY24	*MAT***a**/*MATα sub1-1/cup9Δ::LEU2 ubr1Δ::HIS3/ubr1Δ::HIS3 trp1-Δ63/trp1-Δ63 ura3-52/ura3-52 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 lys2-801/lys2-801*	Produced by mating CBY15 and CBY17

[a]Johnson *et al.* (1992, 1995). A gift from E.Johnson, the Rockefeller University, New York, NY 10021-6399, USA.
[b]Chen *et al.* (1993). A gift from M.Hochstrasser, Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL 60637, USA.
[c]A gift from S.Jentsch, ZMBH, Universität Heidelberg, 69120 Heidelberg, Germany.
[d]Dohmen *et al.* (1990).
[e]A gift from K.Madura, Department of Biochemistry, UMDNJ-Johnson Medical School, Piscataway, NJ 08854, USA.
[f]A gift from J.Dohmen, Heinrich-Heine-Universität, Institut für Mikrobiologie, 40225 Düsseldorf, Germany.
[g]A gift from R.Deshaies, Division of Biology, Caltech, Pasadena, CA 91125, USA.
[h]A gift from S.Grigoryev, Department of Biology, University of Massachusetts, Amherst, MA 01003, USA.

disruption allele was used to replace the wild-type *CUP9* alleles of JD52 (wild-type) and JD55 (*ubr1*Δ) by homologous recombination (Rothstein, 1991), generating strains CBY19 and CBY17, respectively.

CUP9-expressing plasmids

The plasmid pCB116, which expressed Cup9p-FLAG from the P_{GAL1} promoter, was constructed by subcloning an ~1 kb *Bam*HI–*Eco*RI fragment containing the *CUP9-FLAG* ORF into the *Bam*HI–*Eco*RI site(s) of p416GAL1 (Mumberg *et al.*, 1994). The *CUP9-FLAG*-containing fragment was constructed by PCR amplification of the *CUP9* ORF of plasmid pCB111 using primers PCB1 (5′-CGCGGATCCGAATAGTTACATTCGAAGATG-3′) and PCB6 (5′-CCGGAATTCTCATTTATCATCATCGTCTTTGTAATCATTCATATCAGGGTTGGATAG-3′), resulting in the addition of the 8-residue FLAG epitope, DYKDDDDK, to the C-terminus of Cup9p. pCB111 was constructed by subcloning the ~2.8 kb *Hin*dIII–*Kpn*I fragment of the *CUP9*-containing pSUB1-1 (see above) into *Hin*dIII–*Kpn*I-cut pRS316 (Sikorski and Hieter, 1989). pCB202 was constructed by subcloning an ~1 kb fragment containing the P_{CUP9} promoter into *Hin*dIII–*Bam*HI-cut pCB201. [The ~1 kb fragment was produced by PCR from pCB111 using primers PCB8

(5′-GTGTTAGTAAGCTTGTAAAGGAATGCACGTATT-3′) and PCB9 (5′-CCCGCGGATCCGCATGCAACTATTCTCGAAGGTTGT-3′).]

pCB200 (*ARS-CEN*, *LEU2*) and pCB201 (2μ, *LEU2*) were constructed by replacing the 517 bp *Sca*I–*Eco*RI fragment of pBR322 (Ausubel *et al.*, 1992) with, respectively, the 3822 bp *Sca*I–*Nae*I fragment of pRS415 (Sikorski and Hieter, 1989) and the 4650 bp *Sca*I–*Nae*I fragment of pRS425 (Christianson *et al.*, 1992).

The plasmid pCB209 (2μ, *LEU2*), which expressed *CUP9* from the P_{CUP9} promoter, was constructed by replacing the *Sph*I–*Sal*I fragment of pCB202 with an ~1 kb fragment containing the *CUP9* ORF that was produced by PCR from pCB111, using primers PCB10 (5′-CCCGCGGATCCGCATGCGAAGATGAATTATAACTGC-3′) and PCB12 (5′-CCCGCGCGGTCGACCTCAATTCATATCAGGGTTGGATAG-3′). pCB210 (*ARS-CEN*, *LEU2*) that expressed Cup9p-FLAG from the P_{CUP9} promoter was constructed by replacing the *Sph*I–*Sal*I fragment of pCB202 with an ~1 kb fragment containing the *CUP9-FLAG* ORF, which was produced from pCB111 using primers PCB10 (see above) and PCB13 (5′-CCCGCGCGGTCGACCTCATTTATCATCATCGTCTTTGTA-ATCATTCATATCAGGGTTGGATAG-3′), yielding pCB211. The ~2 kb *Hin*dIII–*Sal*I fragment of pCB211 containing P_{CUP9} and the *CUP9-FLAG* ORF was subcloned into pCB200, yielding pCB210.

Plasmid pCB120, expressing GST-Cup9p-ha_2 from the P_{GAL1} promoter, was constructed by subcloning the ~1.6 kb *Xba*I–*Eco*RI fragment, containing the *GST-CUP9-ha*$_2$ ORF, into the *Xba*I–*Eco*RI site(s) of p416GAL1. The *Xba*I–*Eco*RI fragment was produced by PCR amplification of the *GST-CUP9-ha*$_2$ ORF of pGEX-2T–CUP9-ha_2, using the primers PCB3 (5′-CCGGAATTCTCAAGCGTAATCTGGAACATC-GTATGGGTAAGCGTAATCTGGAACATCGTATGGGTAATTCATA-TCAGGGTTGGATAG-3′) and PCB5 (5′-TGCTCTAGAACAGT-ATTCATGTCCCCTATA-3′). pGEX-2T–CUP9-ha_2 was constructed by subcloning an ~1 kb fragment containing the *CUP9-ha*$_2$ ORF into the *Bam*HI–*Eco*RI site(s) of pGEX-2T (Pharmacia), resulting in an in-frame fusion of the sequence encoding 26 kDa glutathione *S*-transferase (GST) domain of *Saccharomyces japonicum* (Smith and Johnson, 1988) to the second codon of *CUP9*. The *CUP9-ha*$_2$-containing fragment was produced by PCR amplification of the *CUP9* ORF of pCB111 using the primers PCB3 (see above) and PCB4 (5′-CGCGGATCCAATTAT-AACTGCGAAATACAAAAC-3′). This step added to the C-terminus of Cup9p a sequence encoding a tandem repeat of the 9-residue sequence YPYDVPDYA, derived from hemagglutinin (ha) of influenza virus.

Northern hybridization

RNA was isolated from *S.cerevisiae* as described (Schmitt *et al.*, 1990). Electrophoresis of the RNA samples was carried out on a formaldehyde RNA gel (Ausubel *et al.*, 1992). An ~50-μg RNA sample was loaded on a 1% agarose gel containing 1× MOPS buffer, 0.74% (v/v) formaldehyde, 1.9 mg/ml iodoacetamide and 0.5 μg/ml ethidium bromide. Electrophoresis was carried out in 1× MOPS buffer at 5 V/cm. RNA was transferred to BrightStar-Plus membrane (Ambion) using TurboBlotter (Schleicher & Schuell) and Ambion RNA transfer buffer. RNA was crosslinked to the air-dried membranes using 254 nm light (Ausubel *et al.*, 1992).

DNA probes were prepared by the random priming method (Ausubel *et al.*, 1992) using [^{32}P]dCTP and a DNA labeling kit (Pharmacia). Hybridization was carried out for 8–16 h at 42°C in Prehybridization/Hybridization Solution (Ambion). Filters were washed according to the manufacturer's protocol and subjected to autoradiography.

Gel shift assay

PCR was used to extend the Cup9p ORF with a sequence encoding Ser-Gly-Gly-Thr-His_6, yielding Cup9p-H_6, and to engineer flanking restriction sites (*Nde*I and *Bam*HI) for insertion into pET-11c (Novagen). Cup9p-H_6 was overexpressed in *E.coli* BL21 (DE3) (Novagen) (Ausubel *et al*., 1992) and purified on a 3 ml Ni-NTA column (Qiagen), using a linear gradient of imidazole. Cup9p-H_6 eluted at ~0.25 M imidazole (~90% pure at this step); it was dialyzed at 4°C against 10% glycerol, 0.1 M KCl, 1 mM EDTA, 0.5 mM dithiothreitol, 20 mM HEPES, pH 7.9, and then snap-frozen in multiple samples in liquid N_2, and stored at –80°C. The proximal (–1 to –447) and distal (–448 to –897) *PTR2* promoter probes for the gel shift assay were constructed by PCR amplification in the presence of [α-^{32}P]dCTP, and were purified using spin columns (Qiagen). The gel shift reactions (20 μl) contained 50 μg/ml poly-dI·dC (Pharmacia); ~1.5 μg/ml (500 c.p.m.) DNA probe; 1 mg/ml acetylated serum albumin (New England Biolabs) and either 0.1, 0.5 or 1 μg/ml of Cup9p-H_6 in 10% glycerol, 0.1 M KCl, 2.5 mM $MgCl_2$, 1 mM EDTA, 20 mM HEPES, pH 7.9. The samples were incubated for 30 min at room temperature, then loaded onto a 4% polyacrylamide gel (40:1, acrylamide:*bis*-acrylamide) in 0.5× TBE (Ausubel *et al*., 1992), and electrophoresed at 10 V/cm for 3 h at 4°C, followed by autoradiography.

Pulse–chase analysis of Cup9p

One hundred ml cultures of *S.cerevisiae* JD52 (*UBR1*) and JD55 (*ubr1*Δ) carrying either pCB210 (expressing Cup9p-FLAG from the *CUP9* promoter) or pCB200 (vector alone) were grown to an A_{600} of ~1 in SD(–Leu) medium. Cells (50 A_{600} units total) from each of the four cultures were gently pelleted by centrifugation, washed with 5 ml of SD(–Leu), pelleted again, resuspended in 2 ml of SD(–Leu), and incubated at 30°C for 10 min. Each sample was labeled for 5 min with 1.4 mCi of [^{35}S]methionine/cysteine (EXPRESS, New England Nuclear) at 30°C, followed by pelleting in a microfuge for ~15 s. The cells were resuspended in 2.6 ml of SD(–Leu), 5 mM L-methionine, 5 mM L-cysteine, and incubated at 30°C. Samples of 0.5 ml were withdrawn during the incubation, pelleted and resuspended in 0.15 ml of 0.5 M NaCl-Lysis Buffer (1% Triton X-100, 0.5 M NaCl, 5 mM EDTA, 50 mM Na-HEPES, pH 7.5) containing a mixture of protease inhibitors (Ghislain *et al.*, 1996). Glass beads (0.5 mm) were added, and cells were disrupted by vortexing (six times, for 30 s each, with 1 min incubations on ice in between), followed by the adjustment of NaCl concentration to 0.15 M through the addition of 75 mM NaCl-Lysis Buffer, further vortexing for 30 s, and centrifugation at 12 000 *g* for 10 min. The volumes of supernatants were adjusted to equalize the amounts of 10% trichloroacetic acid-insoluble ^{35}S. Cup9p-FLAG was immunoprecipitated by the addition of 20 μl of the monoclonal anti-FLAG M2 antibody conjugated to agarose beads (Kodak). Suspensions were incubated at 0°C for 1 h, with rotation, then centrifuged at 12 000 *g* for 30 s, and washed four times with 0.8 ml of 0.15 M NaCl-Lysis Buffer. The pellets were resuspended in SDS-sample buffer, heated at 100°C for 3 min, and subjected to SDS–12% PAGE, followed by autoradiography and quantitation using a PhosphorImager (Molecular Dynamics).

Pulse–chase analysis of GST-Cup9p-ha_2 was carried out as described by Bartel *et al.* (1990). Approximately 10 A_{600} units of galactose-induced cells were labeled for 5 min with 0.3 mCi of [^{35}S]EXPRESS in 400 μl SM–galactose (–Ura) at 30°C. The cells were then transferred to microfuge tubes, pelleted and resuspended in 500 μl of SD (–Ura), 5 mM L-methionine, 5 mM L-cysteine. Samples of 0.1 ml were withdrawn during the incubation, pelleted and lysed as above. The ^{35}S-labeled GST-Cup9p-ha_2 was purified using glutathione–agarose beads (Sigma) which had been blocked with bovine serum albumin (BSA; 10 mg/ml). Twenty μl of glutathione–agarose beads were added to each sample and the suspensions were incubated at 0°C for 60 min, with rotation, followed by washes and electrophoretic analyses as described for Cup9p-FLAG.

Purification and N-terminal sequencing of Cup9p-FLAG

Four 2-l cultures of JD55 (*ubr1*Δ) carrying pCB116 that expressed Cup9p-FLAG from the *GAL1* promoter were grown under selection in SM–raffinose to an A_{600} of ~0.8, followed by transfer to SM–galactose and incubation at 30°C for 3 h. Longer induction times resulted in a Cup9p-mediated cytotoxicity and lower yields of Cup9p-FLAG. The cells (~1×10^{11}) were harvested and lysed at 4°C as described by Burgers (1995). The extract was fractionated by precipitation with 0.4% Polymin P (Sigma) and then further by precipitation with 48% saturated ammonium sulfate. The pellet was dissolved in 3 ml of TBS buffer (0.15 M NaCl, 50 mM Tris–HCl, pH 7.5), and passed through Sephadex G-25 in TBS. The resulting sample (8.3 ml, ~70 mg/ml of protein) was applied to a column (1 ml) of the monoclonal anti-FLAG M2 antibody (Kodak). The column was washed three times with 10 ml of TBS, and Cup9p-FLAG was eluted by the addition of five 1-ml samples of TBS containing, respectively, 50, 100, 100, 200 and 200 μg/ml of the FLAG peptide (Kodak). Peak Cup9p-FLAG fractions (detected by immunoblotting) were concentrated by partial lyophilization, followed by precipitation with methanol (Wessel and Flügge, 1984) in the presence of human insulin (Sigma, 0.3 mg/ml) as a carrier. The resulting sample was fractionated by SDS–12% PAGE and electroblotted onto Pro-Blot membrane (Perkin-Elmer). After a brief staining with Coomassie, the band of the 37 kDa Cup9p-FLAG (~15 pmol) was excised from the membrane. Half of the sample was used to determine the amino acid composition; the other half was subjected to N-terminal sequencing for seven cycles, using the Applied Biosystems 476A protein sequencer at the Caltech Microchemistry Facility.

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