

Amino Acids Induce Peptide Uptake via Accelerated Degradation of CUP9, the Transcriptional Repressor of the PTR2 Peptide Transporter*

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Multiple pathways link expression of PTR2, the transporter of di- and tripeptides in the yeast *Saccharomyces cerevisiae*, to the availability and quality of nitrogen sources. Previous work has shown that induction of PTR2 by extracellular amino acids requires, in particular, SSY1 and PTR3. SSY1 is structurally similar to amino acid transporters but functions as a sensor of amino acids. PTR3 acts downstream of SSY1. Expression of the PTR2 peptide transporter is induced not only by amino acids but also by dipeptides with destabilizing N-terminal residues. These dipeptides bind to UBR1, the ubiquitin ligase of the N-end rule pathway, and allosterically accelerate the UBR1-dependent degradation of CUP9, a transcriptional repressor of PTR2. UBR1 targets CUP9 through its internal degron. Here we demonstrate that the repression of PTR2 by CUP9 requires TUP1 and SSN6, the corepressor proteins that form a complex with CUP9. We also show that the induction of PTR2 by amino acids is mediated by the UBR1-dependent acceleration of CUP9 degradation that requires both SSY1 and PTR3. The acceleration of CUP9 degradation is shown to be attained without increasing the activity of the N-end rule pathway toward substrates with destabilizing N-terminal residues. We also found that GAP1, a general amino acid transporter, strongly contributes to the induction of PTR2 by Trp. Although several aspects of this complex circuit remain to be understood, our findings establish new functional links between the amino acids-sensing SPS system, the CUP9-TUP1-SSN6 repressor complex, the PTR2 peptide transporter, and the UBR1-dependent N-end rule pathway.

Biological processes addressed by this study include the mechanisms and regulation of peptide import. Peptides can serve as a source of amino acids and nitrogen in all organisms. The import of di- and tripeptides (di/tripeptides) in the yeast

Saccharomyces cerevisiae has been shown to be regulated by the N-end rule pathway, one proteolytic pathway of the ubiquitin (Ub)⁴-proteasome system (1–4). The N-end rule relates the *in vivo* half-life of a protein to the identity of its N-terminal residue (reviewed in Refs. 5–7). Although prokaryotes lack the Ub system, they still contain the N-end rule pathway, albeit Ub-independent versions of it (6, 8, 9). In eukaryotes, this pathway recognizes several kinds of degradation signals (degrons), including a set called N-degrons (5–7, 10). Specific N-degrons that are active (recognized) in a cell give rise to that cell's N-end rule. The determinants of an N-degron in a substrate protein are a destabilizing N-terminal residue that bears the unmodified N-terminal amino group, a substrate's internal Lys residue (the site of formation of a poly-Ub chain), and a nearby conformationally disordered region (11–13).

The N-end rule has a hierarchic structure. In eukaryotes, N-terminal Asn and Gln are tertiary destabilizing residues in that they function through their enzymatic deamidation, to yield the secondary destabilizing N-terminal residues Asp and Glu (Ref. 7, and references therein). Destabilizing activity of N-terminal Asp and Glu requires their conjugation to Arg, one of the primary destabilizing residues, by the ATE1-encoded Arg-tRNA-protein transferase (arginyl-transferase or R-transferase) (14–18). In mammals and other eukaryotes that produce nitric oxide (NO), the set of arginylated residues contains not only Asp and Glu but also N-terminal Cys, which is arginylated after its oxidation to Cys-sulfinate or Cys-sulfonate (14). The *in vivo* oxidation of N-terminal Cys requires NO, as well as oxygen (O₂) or its derivatives (15, 18). The N-end rule pathway is thus a sensor of NO, through the ability of this pathway to destroy proteins with N-terminal Cys, at rates controlled by NO, O₂, and their derivatives.

E3 Ub ligases of the N-end rule pathway are called N-recognins (7, 10, 19). They recognize (bind to) primary destabilizing N-terminal residues. (The term "Ub ligase" denotes either an E2–E3 holoenzyme or its E3 component.) At least four N-recognins, including UBR1, mediate the N-end rule pathway in mammals and other multicellular eukaryotes (7). The known N-recognins share a ~70-residue motif called the UBR box. Mouse UBR1 and UBR2 are sequelogous (similar in sequence) 200-kDa RING-type E3 Ub ligases that are 47% identical. Sev-

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⁴ The abbreviations used are: Ub, ubiquitin; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; β gal, β -galactosidase; UPR, Ub-protein-reference.

eral other mammalian N-recognins, either confirmed or putative ones, are HECT-type or SCF-type Ub ligases that share the UBR motif with the RING-type UBR1 and UBR2 but are largely nonsequelous to them otherwise (20, 21).⁵

The functions of the N-end rule pathway include: (i) the sensing of heme, owing to inhibition, in both yeast and mammals, of the ATE1 R-transferase by heme (Fe^{3+} -heme), which also inhibits N-recognins, the latter at least in yeast; (ii) the sensing of NO and O_2 , and the resulting control of signaling by transmembrane receptors, through the conditional, NO/ O_2 -mediated degradation of G-protein regulators RGS4, RGS5, and RGS16; (iii) regulation of import of short peptides, through the degradation, modulated by peptides, of CUP9, the repressor of import; (iv) fidelity of chromosome segregation, through degradation of a separase-produced cohesin fragment; (v) regulation of apoptosis, through degradation of a caspase-processed inhibitor of apoptosis; (vi) a multitude of processes mediated by the transcription factor c-FOS, a conditional substrate of the N-end rule pathway; (vii) regulation of the human immunodeficiency virus replication cycle, through degradation of human immunodeficiency virus integrase; and (viii) regulation of meiosis, spermatogenesis, neurogenesis, and cardiovascular development in mammals, and leaf senescence in plants (Refs. 2–4, 7, 14, 15, 17–19, 23–25, and references therein). Mutations in human UBR1, one of the E3s of the N-end rule pathway, are the cause of Johansson-Blizzard syndrome, which comprises mental retardation, physical malformations, and severe pancreatitis (26).

The N-end rule pathway of *S. cerevisiae* is mediated by a single N-recognin, UBR1, a 225-kDa sequelog of mammalian UBR1 and UBR2 (2, 10, 27). *S. cerevisiae* UBR1 contains at least three substrate-binding sites. The type-1 site is specific for basic N-terminal residues of polypeptides (Arg, Lys, and His). The type-2 site is specific for bulky hydrophobic N-terminal residues (Trp, Phe, Tyr, Leu, and Ile). The third binding site of UBR1 recognizes an internal (non-N-terminal) degron in target proteins. The third binding site of UBR1 is autoinhibited but can be allosterically activated through a conformational change that is caused by the binding of short peptides, such as dipeptides, to the other, type-1 and type-2, binding sites of UBR1. The known substrate of the third binding site of UBR1 is CUP9 (1–3), a homeodomain protein and transcriptional regulator, largely a repressor, of more than 30 genes in *S. cerevisiae*.⁶ The regulon of CUP9 includes *PTR2*, a gene encoding the main transporter of di/tripeptides (28, 29). The reversal of UBR1 autoinhibition by imported di/tripeptides with destabilizing N-terminal residues accelerates the UBR1-dependent ubiquitylation of CUP9, leads to its faster degradation, and thereby

causes a derepression of *PTR2*. The resulting positive-feedback circuit allows *S. cerevisiae* to detect the presence of extracellular peptides and to react by increasing their uptake (2, 3).

The evolution of peptide-import circuits is under conflicting selective pressures, because the ability of a cell to import peptides confers both a benefit (utilization of peptides as food) and a vulnerability to toxins that resemble short peptides. Although *PTR2* is the major transporter of di/tripeptides in *S. cerevisiae*, some di/tripeptides can also be imported (with a low efficacy) by *DAL5*, whose major function is the import of other nitrogen sources, such as allantoate and ureidosuccinate (4). Another set of *S. cerevisiae* peptide transporters comprises *OPT1* (*HGT1*) and *OPT2*, which have partially overlapping functions, do not import di/tripeptides, but can import peptides of 4–5 residues. In addition, *OPT1* (*HGT1*) is a high affinity importer of glutathione, a “noncanonical” tripeptide (Ref 30, and references therein). Similarly to the *PTR2* transporter of di/tripeptides, the expression of *OPT2* is down-regulated by CUP9, whereas the expression of *OPT1* (*HGT1*) is independent of CUP9 (30). In addition to *PTR2* and *OPT2*, the N-end rule pathway also controls the expression of *DAL5*, but in a manner opposite to that of the other two transporters: whereas CUP9 is a transcriptional repressor of *PTR2* and *OPT2*, CUP9 apparently up-regulates the expression of *DAL5* (28, 30). It is unknown whether CUP9 down-regulates a repressor of *DAL5* or whether CUP9 acts, in the context of *DAL5*, as a transcriptional activator.

The induction of the *PTR2* peptide transporter by di/tripeptides, a process controlled by the UBR1-CUP9 circuit (3), is just one of regulatory inputs that couple *PTR2* expression to the availability and quality of nutrients. For example, *PTR2* expression is down-regulated by certain nitrogen sources, including ammonia, but not by other nitrogen sources, such as urea and allantoin (31). The underlying systems, including the N-end rule pathway, ensure that a cell does not waste resources synthesizing large amounts of the *PTR2* transporter in the absence of extracellular peptides, or when a more efficacious nitrogen source, such as ammonia, is present.

PTR2 is also induced by extracellular amino acids, particularly leucine or tryptophan (32). This response is likely to be adaptive in natural habitats, because the amino acids that *S. cerevisiae* (a scavenging heterotroph) encounters outside the laboratory tend to be the breakdown products of peptides and thus signify a likely presence of di/tripeptides. In addition, it has not been precluded that the *S. cerevisiae* *PTR2* transporter might itself import amino acids, in addition to peptides. For example, At*PTR2* and At*CHL1*, members of the *PTR2* family in the plant *Arabidopsis thaliana*, transport histidine and nitrate, respectively (Ref. 33, and references therein). Either of the above possibilities may underlie the fact that amino acids regulate *PTR2* expression.

Extracellular amino acids regulate *PTR2* through the SPS (*SSY1-PTR3-SSY5*) pathway (34–40). *SSY1*, an integral membrane protein and a sensor of amino acids, is a sequelog of amino acid transporters but does not function as a transporter (36, 37, 41), a disposition that recurs with other nutrient sensors as well (37, 42). Both the inferred design of *SSY1* and experimental evidence suggest that it is the concentration ratio of an amino acid across the plasma membrane, rather than the abso-

⁵ A note on terminology: “sequelog” and “spalog” denote, respectively, a sequence that is similar, to a specified extent, to another sequence, and a three-dimensional structure that is similar, to a specified extent, to another three-dimensional structure (22). Besides their usefulness as separate terms for sequence and spatial similarities, the rigor-conferring advantage of sequelog and spalog is their *evolutionary neutrality*, in contrast to interpretation-laden terms such as “homolog,” “ortholog,” and “paralog.” The latter terms are compatible with the sequelog/spalog terminology and can be used to convey understanding about functions and common descent, if this (additional) information is available.

⁶ C. S. Hwang and A. Varshavsky, unpublished data.

lute concentration of extracellular amino acid that determines the signaling output by SSY1 (36). Activated SSY1 induces expression of a regulon that includes the PTR2 peptide transporter and amino acid transporters such as AGP1, BAP2, BAP3, TAT1, TAT2, and GNP1 (38). PTR3 and SSY5 are peripheral membrane proteins associated with SSY1 (41). SSY5 is a protease regulated, in particular, by PTR3. SSY5 can cleave, and thereby activate, the latent (conditionally cytosolic) transcriptional activators STP1 and STP2, leading to their import into the nucleus and the induction of genes that encode, in particular, amino acid transporters (34–37, 39, 40, 43).

In the present work, we show that an extracellular amino acid such as Trp acts via the SPS system to induce the PTR2-mediated import of di/tripeptides through the acceleration of degradation of CUP9 (the repressor of import) by the UBR1-dependent N-end rule pathway. The bulk of this effect of Trp on the rate of CUP9 degradation requires both SSY1 and PTR3. At the same time, no significant activation of the N-end rule pathway toward substrates with destabilizing N-terminal residues (*i.e.* toward substrates with N-degrons) was observed under these conditions, suggesting a differential regulation of three substrate-binding sites of the UBR1 Ub ligase. We also show that the repression of *PTR2* by CUP9 requires the global corepressors TUP1 and SSN6, and that GAP1, a general amino acid transporter, strongly contributes to the induction of *PTR2* by Trp. Although several aspects of this complex circuit remain to be understood, our findings establish new functional links between the amino acids-sensing SPS system, the CUP9-TUP1-SSN6 repressor complex, the *PTR2* peptide transporter, and the UBR1-dependent N-end rule pathway.

EXPERIMENTAL PROCEDURES

Yeast Strains, Plasmids, and Genetic Techniques—The *S. cerevisiae* strains used in this study are described in Table 1. AVY24 and AVY25 were constructed in the background of strain RJD347 (*MAT α ura3–52*; a gift from Dr. R. Deshaies, California Institute of Technology). A PCR-based deletion strategy (44) was employed, using DNA fragments encoding the *myc*₃-*URA3*-*myc*₃ cassette to precisely delete *SSY1* and *PTR3* open reading frames (*myc*₃ denotes 3 repeats of a nucleotide sequence encoding the *myc* epitope, flanked on either side by 50 bp of sequences identical to the sequences of either the *SSY1* or the *PTR3* gene, respectively). This technique was employed to produce deletions of the *SSY1* and *PTR3* open reading frames. We then selected for recombination within the *myc*₃-*URA3*-*myc*₃ module using 5-fluoroorotic acid, to produce strains AVY27 (*MAT α ura3–52 ssy1 Δ ::myc₃*) and AVY28 (*MAT α ura3–52 ptr3 Δ ::myc₃*), and verified the identity of these strains by Southern hybridization (data not shown). AVY26 (*MAT α ura3–52 ubr1 Δ ::HisG*) was also a derivative of RJD347 (Table 1). It was constructed using the same PCR-based deletion strategy (44), except that a *HisG-URA3-HisG* cassette (see below) was used. Strains AVY30 (*MAT α leu2–3,112 ubr1 Δ ::LEU2*), AVY31 (*MAT α leu2–3,112 cup9 Δ ::LEU2*), and AVY32 (*MAT α LEU2*) (Table 1) were derivatives of RJD350 (*MAT α leu2–3,112*) (a gift from Dr. R. Deshaies).

PCR with *S. cerevisiae* genomic DNA was used to produce a fragment containing *SSN6* flanked by 901 bp and 400 bp of

upstream and downstream sequences, respectively. The fragment was ligated to NotI/EcoRI-cut pRS314 (45). The same procedure was used to construct a fragment containing *TUP1* flanked by 459 bp and 400 bp of upstream and downstream sequences, respectively, followed by ligation to SpeI/Clal-cut pRS416 (45). pSsn6 Δ , which carried *ssn6 Δ ::HisG-URA3-HisG*, was constructed by subcloning the *SSN6*-containing NotI-EcoRI fragment into NotI/EcoRI-cut Bluescript (Stratagene), producing pBlueSSN6. Using PCR, a deletion (from 50 bp upstream of the *SSN6* start codon to position 2,585 of its open reading frame) was introduced, while simultaneously creating an EclXI site at the upstream breakpoint of the deletion. The EclXI-PstI fragment of pAS315 (a gift from Dr. A. Sil, University of California, San Francisco), containing *HisG-URA3-HisG*, was then inserted. *In vivo* integration of the NotI-EcoRI fragment of the resulting plasmid (pSsn6 Δ), carrying *ssn6 Δ ::HisG-URA3-HisG*, produced a deletion of *SSN6* that spanned the region from –50 to 2585, relative to the start codon. pTup1 Δ , which carried *tup1 Δ ::HisG-URA3-HisG*, was constructed similarly, using the SpeI-Clal fragment containing *TUP1* (from pTUP1), its subcloning into pBluescript (thus yielding pBlueTUP1), and PCR to produce a deletion allele of *TUP1* (from 7 bp upstream of its start codon to position 2016 of the *TUP1* open reading frame) that contained a (PCR-added) BamHI site at the deletion breakpoint. The BamHI-EcoRI fragment of pAS135 was then inserted into pBlueTUP1. *In vivo* integration of the SpeI-XhoI fragment of the resulting plasmid (carrying *tup1 Δ ::HisG-URA3-HisG*) produced a deletion of *TUP1*, from –7 to +2016, relative to the start codon. To select for recombination/excision within the integrated *HisG-URA3-HisG* cassette and thereby to produce *ssn6 Δ ::HisG* and *tup1 Δ ::HisG* alleles, 5-fluoroorotic acid-resistant yeast colonies were isolated, and the identities of strains were verified by Southern hybridization (data not shown). This procedure was performed in the JD52 background to construct strains AVY60 and AVY61; in the JD55 (*ubr1 Δ*) background (Table 1) (23) to construct AVY62 and AVY63; in the AVY50 background (1) to construct AVY64 and AVY65; and in the AVY51 background (1) to construct AVY66 and AVY67 (Table 1).

SSN6 tagged at the C terminus with two copies of the *myc* epitope (SSN6_{myc2}) and expressed from its own promoter on a high copy (2 μ) plasmid was a gift from Dr. R. Zitomer (State University of New York, Albany). For plasmid-based complementation assays, plasmids encoding SSN6 (pSSN6) and TUP1 (pTUP1) were constructed using standard techniques. ^fDHFR-Ub^{K48R}-CUP9_{NSF} was expressed from the P_{MET25} promoter in the plasmid pMET416UPRCUP9_{NSF}, based on the low copy (*CEN*) vector p416MET25 (3). The previously described (27) pUb23-X plasmids expressed X- β -galactosidase reporter proteins (derived from the corresponding Ub-X- β -galactosidase fusions; X = Met, Ala, His, or Tyr) from the P_{GALI} promoter in a high copy vector.

Coimmunoprecipitation, Pulse-chase, β -Galactosidase, and Northern Hybridization Assays—For CUP9-SSN6 coimmunoprecipitation assays, *S. cerevisiae* carrying the indicated plasmids were grown to A₆₀₀ of ~1 in SD medium (12) containing auxotrophic supplements. Cells from a 10-ml culture were harvested by centrifugation, washed in 1 ml of water, and resus-

pended in 0.8 ml of ice-cold buffer C (0.25 M NaCl, 1 mM EDTA, 50 mM HEPES, pH 7.5) containing ovalbumin at 1 mg/ml. Cells were disrupted by vortexing with 0.5 ml (packed volume) of 0.5-mm glass beads in the presence of protease inhibitors (Roche Applied Science). Extracts were prepared and immunoprecipitations were carried out as described (46), with two modifications. First, after incubation of an extract with anti-FLAG beads (Sigma) for 45 min at 4 °C, the resulting immunoprecipitate was washed once with 1 ml of buffer C, and then twice more with 1 ml of buffer C lacking ovalbumin. Second, immunoprecipitates were eluted from anti-FLAG beads through a 15-min incubation, at room temperature, with the FLAG peptide at 0.5 mg/ml, in buffer C lacking ovalbumin. An equal volume of 2× SDS-PAGE sample buffer was added, followed by SDS-13% PAGE and immunoblotting with anti-*myc* antibody (Covance, Berkeley, CA), followed by a secondary horseradish peroxidase-conjugated goat anti-mouse antibody (Bio-Rad) at 1:1,000 dilution, and thereafter a detection of the latter antibody using ECL assay (GE Healthcare, Piscataway, NJ).

For pulse-chase assays, *S. cerevisiae* was grown to A_{600} of ~0.8 in SHM medium (2% glucose, 0.1% allantoin, and 0.17% yeast nitrogen base without amino acids and without ammonium sulfate) that either contained or lacked tryptophan (98 μ M, 20 μ g/ml), an inducing amino acid (32). Cells were harvested, washed in 0.8 ml of SHM \pm Trp, resuspended in 0.4 ml of the same medium, and labeled for 5 min at 30 °C with 0.16 mCi of 35 S-EXPRESS (PerkinElmer Life Sciences). Thereafter cells were pelleted, resuspended in fresh SHM \pm tryptophan containing 4 mM L-methionine and 2 mM L-cysteine, and incubated further at 30 °C. Samples of 0.1 ml were taken at indicated time points and transferred to chilled tubes, each containing 0.5 ml of 0.5-mm glass beads, 0.7 ml of ice-cold lysis buffer (1% Triton X-100, 0.15 M NaCl, 5 mM EDTA, 50 mM HEPES, pH 7.5), and a mixture of protease inhibitors (final concentrations: 1 mM freshly prepared phenylmethylsulfonyl fluoride, 2 μ g/ml aprotinin, 0.5 μ g/ml leupeptin, and 0.7 μ g/ml pepstatin). Extracts were prepared, and immunoprecipitations were carried out as described previously (46), using anti-FLAG M2 beads (Sigma). Immunoprecipitated proteins were fractionated by SDS-13% PAGE, followed by autoradiography. Some of the pulse-chase assays were carried out in the presence of cycloheximide (at 0.2 mg/ml) during the chase.

For β -galactosidase (β gal) activity assays, *S. cerevisiae* carrying a pUB23-X plasmid (27) and expressing, from the P_{GAL1} promoter, an X- β gal test protein (derived from Ub-X- β -galactosidase, with X = Met, Ala, His, or Tyr) (47) were grown in SHM medium overnight. Cultures were diluted to A_{600} of ~0.1 in SHM containing 2% galactose instead of glucose and either containing or lacking 98 μ M Trp, followed by growth until A_{600} of ~0.5. Cell extracts were thereafter prepared and processed for assays with *o*-nitrophenyl *o*-nitrophenyl- β -D-galactopyranoside as previously described (27). For Northern analyses of *PTR2* induction by amino acids, cells were grown to A_{600} of ~0.6 in SHM containing or lacking 98 μ M (20 μ g/ml) Trp. Total RNA was prepared as described previously (16), using an RNeasy Mini Kit (Qiagen, Valencia, CA). Samples containing 25 μ g of RNA were electrophoresed in 1% formaldehyde-agar-

ose gels, followed by blotting for Northern analysis (3). Northern assays of the effects of deletions of specific genes on *PTR2* expression were carried out identically, in the absence of added Trp.

RESULTS AND DISCUSSION

Repression of PTR2 Transcription by CUP9 Involves the CUP9-TUP1-SSN6 Corepressor Complex—UBR1, the E3 Ub ligase of the *S. cerevisiae* N-end rule pathway, regulates the import of peptides by controlling, through degradation of the transcriptional repressor CUP9, the expression of the peptide transporter *PTR2* (1). *PTR2* mRNA was originally shown to be absent in *ubr1* Δ cells (1, 48), consistent with a mechanism in which a normally short-lived repressor of *PTR2* becomes long-lived in the absence of UBR1, thereby increasing in concentration and shutting down *PTR2* transcription. CUP9 was identified using a bypass screen for mutations that restored dipeptide uptake and *PTR2* expression in *ubr1* Δ cells (1).

Further analysis, described below, of our collection of bypass mutants identified additional isolates that did not belong to the *CUP9* complementation group. Many of these non-*cup9* mutants (Fig. 1A) exhibited flocculation (aggregation during growth in liquid culture) and had a reduced mating ability. These phenotypes are characteristic of a defect in either *TUP1* or *SSN6*, which encode two “global” corepressors that form a complex interfering with transcription initiation (reviewed in Ref. 49). The TUP1-SSN6 complex, which does not bind to DNA by itself, is recruited to individual promoters through interactions with specific DNA-binding proteins. One such protein is the mating-type regulator *MAT α 2* (50). In the absence of either TUP1 or SSN6, the *MAT α 2*-mediated repression of *MAT α* -specific genes is defective, resulting in mating-defective, pseudodiploid cells (49).

To determine whether the above non-*cup9* isolates (Fig. 1A) were either *tup1* or *ssn6* mutants, they were transformed with low copy plasmids that expressed either *TUP1* or *SSN6* from their natural promoters. These plasmids indeed suppressed the clumpy-growth and peptide-uptake phenotypes of all of the flocculating bypass mutants. The entire collection of our 201 bypass mutants (including *cup9* mutants) was partitioned into complementation groups using a combination of the above plasmid-based complementation tests as well as mating-based complementation assays (the latter could not be comprehensive, owing to mating deficiency of some isolates). The 201 bypass suppressors were found to belong to three complementation groups, defined by *CUP9*, *TUP1*, and *SSN6*. Apart from genes required for cell viability, it is likely that our (nonconditional) screen was an exhaustive one, given the numbers of isolates in each of the three complementation groups thus identified: 104, 67, and 30 isolates of *cup9*, *ssn6*, and *tup1* mutants, respectively (Fig. 1A).

To verify the role of *TUP1* and *SSN6* in regulating peptide uptake, we tested the effects of disrupting either one of these genes on the ability of a *ubr1* Δ strain to import dipeptides. Congenic (*tup1* Δ *ubr1* Δ) and (*ssn6* Δ *ubr1* Δ) double mutants were constructed using homologous recombination in the lysine-auxotrophic (*lys2*) genetic background, and the identities of mutants were verified by Southern hybridization (data

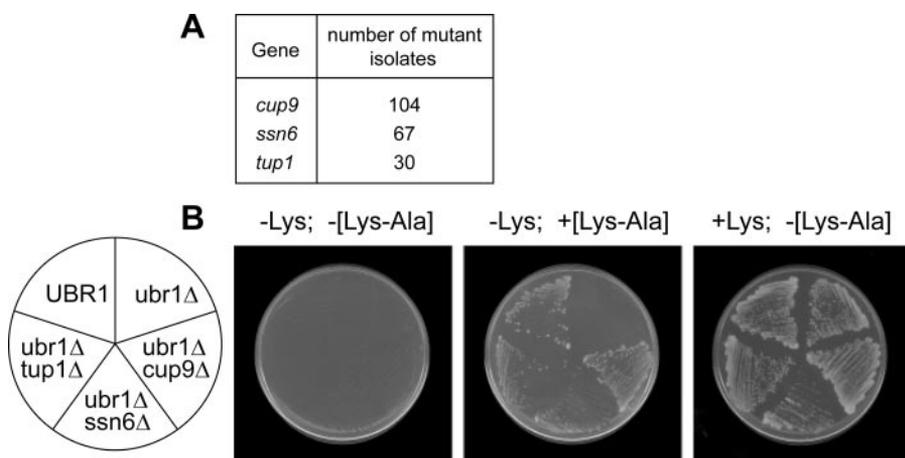


FIGURE 1. Mutations in *SSN6* or *TUP1* rescue the block to dipeptide import in *ubr1Δ* *S. cerevisiae*, which lack the N-end rule pathway. *A*, a collection of 201 mutants in which the ability of a *ubr1Δ* strain to import dipeptides had been restored (*cup9* members of that collection were examined in the earlier study (1)) were classed into complementation groups that included *SSN6* and *TUP1* using plasmid-based or mating-based complementation tests (1). The number of isolates in each complementation group, *cup9*, *tup1*, and *ssn6*, is shown. *B*, *ssn6Δ* and *tup1Δ* restore dipeptide import to a *ubr1Δ* strain. Strains JD52, AVY107, AVY51, AVY62, and AVY63 (Table 1), with relevant genotypes indicated on the plate diagram, were tested for their ability to use the dipeptide Lys-Ala as a source of essential nutrient, lysine. All strains were *lys2* and thus auxotrophic for Lys. Strains were tested for growth on SD medium lacking Lys (*left*), or supplemented with Lys-Ala (*center*), or supplemented with Lys (*right*). Only strains capable of importing dipeptides could form colonies on the [-Lys; +[Lys-Ala]] plate. Deletion of either *SSN6*, *TUP1*, or (as shown previously (1)) *CUP9* restored dipeptide import to a *ubr1Δ* strain (*center plate*).

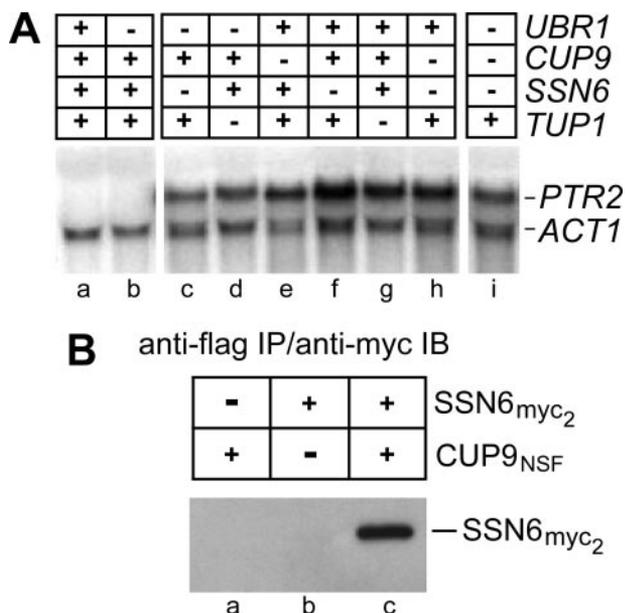


FIGURE 2. *CUP9* represses *PTR2* transcription via *SSN6* and *TUP1*. *A*, *SSN6*, *TUP1*, and *CUP9* are required for repression of the peptide transporter gene *PTR2*. Northern analysis of *PTR2* mRNA in the wild-type strain JD52, and in congenic *ssn6Δ* (AVY60), *tup1Δ* (AVY61), *cup9Δ* (AVY50), and *ubr1Δ* (AVY107) mutants, as well as in various combinations thereof (AVY51 and AVY62–AVY67) (Table 1). The status of each gene (–, deleted; +, wild-type allele) is shown in the upper diagram. *PTR2* and *ACT1* (actin) mRNA are indicated. *S. cerevisiae* strains were grown in SHM without *PTR2*-inducing amino acids or peptides. *B*, coimmunoprecipitation of *myc*-tagged *SSN6* (*SSN6_{myc2}*) with *CUP9_{NSF}*, a FLAG-tagged *CUP9* derivative (see “Results” and “Discussion”). Extracts from cells expressing the indicated combinations of *SSN6_{myc2}* and *CUP9_{NSF}* were immunoprecipitated with a monoclonal anti-FLAG antibody or followed by SDS-PAGE and immunoblotting with a monoclonal anti-*myc* antibody (see “Experimental Procedures”).

not shown). The ability of these Lys-requiring double mutants to import dipeptides was then assayed by monitoring their growth on a minimal medium containing the dipeptide Lys-Ala

as the sole source of Lys (Fig. 1*B*). A *lys2* strain containing the intact *UBR1* gene was able to import sufficient amounts of Lys-Ala dipeptide to support its growth on this medium, whereas a congenic (*ubr1Δ lys2*) strain could not grow under these conditions (it could be rescued by free Lys) (Fig. 1*B*). In contrast to the *ubr1Δ lys2* strain, both *tup1Δ ubr1Δ lys2* and *ssn6Δ ubr1Δ lys2* triple mutants formed colonies on Lys-Ala plates, as did a *cup9Δ ubr1Δ lys2* strain (Fig. 1*B*). Thus, a deletion of either *CUP9*, or *TUP1*, or *SSN6* is each capable of restoring dipeptide uptake to a *ubr1Δ* strain. The slightly smaller colonies formed by the *ssn6Δ ubr1Δ lys2* strain on Lys-Ala plates could be caused by a growth defect of *ssn6Δ* strains, a phenotype that we also observed with the *ssn6Δ ubr1Δ lys2* strain on a control (Lys-supplemented) plate (Fig. 1*B*).

The fact that *cup9*, *tup*, and *ssn6* mutants all bypassed the block to dipeptide uptake in a *ubr1Δ* strain suggested that *CUP9* represses *PTR2* expression by functioning as a part of a *TUP1/SSN6*-containing complex. To address this model, we compared the levels of *PTR2* mRNA in *ssn6Δ* and *tup1Δ* mutants with those in other strains, in the absence of added *PTR2* inducers such as dipeptides or amino acids. In a strain retaining all five of the studied genes, *PTR2*, *UBR1*, *CUP9*, *SSN6*, and *TUP1*, and also in a *ubr1Δ* strain, the level of *PTR2* mRNA was low enough to be undetectable at the sensitivity of Northern assays used (Fig. 2*A*, lanes *a* and *b*; compare with lanes *c–i*). With *ubr1Δ* strains, no *PTR2* mRNA could be detected even by higher sensitivity Northern blots (data not shown), whereas wild-type (*UBR1*) strains, in the absence of either added dipeptides or “inducing” amino acids, contained low but detectable levels of *PTR2* mRNA, as shown previously (1, 3). In contrast, the levels of *PTR2* mRNA were high in the absence of either *CUP9*, or *SSN6*, or *TUP1*, irrespective of the presence or absence of *UBR1* (Fig. 2*A*, lanes *c–i*; compare with lanes *a* and *b*; see also Fig. 3*A*, lane *c* versus lane *i*). Thus, the tight block to dipeptide uptake in a *ubr1Δ* strain, caused by increased levels of the *CUP9* repressor, could be bypassed not only through the removal of *CUP9*, but also through the removal of either *SSN6* or *TUP1* (Fig. 2*A*).

High levels of *PTR2* mRNA in the absence of either *CUP9*, *SSN6*, or *TUP1* (Figs. 2*A* and 3*A*) indicated that each of these proteins was essential for repression of the *PTR2* gene. Both the demonstrated functional dependence of *CUP9* on *SSN6/TUP1* (Figs. 1 and 2*A*) and the close analogy between the homeodomain repressors *CUP9* and *MATα2*, the latter of which is known to interact with *SSN6/TUP1* (see above), suggested that *CUP9* also interacted with *SSN6/TUP1*. To address this experimentally, we carried out a coimmunoprecipitation assay, using

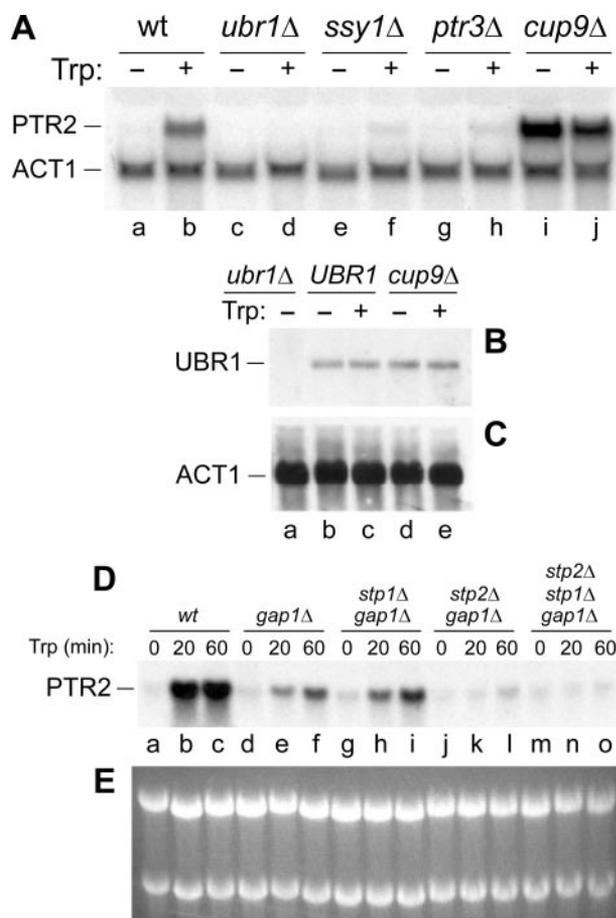


FIGURE 3. Northern analyses of *PTR2* and *UBR1* under different conditions and in different genetic backgrounds. *A*, amino acid-inducible expression of *PTR2* requires *UBR1* and *CUP9*. Congenic strains of the indicated genotype (AVY32, AVY30, AVY24, AVY25, and AVY31) (Table 1) were grown in SHM medium containing or lacking 98 μM (20 $\mu\text{g}/\text{ml}$) tryptophan. Isolated total RNA was analyzed by Northern hybridization with *PTR2* and *ACT1* DNA probes, as indicated. *B* and *C*, levels of *UBR1* expression do not change significantly in the presence versus absence of Trp. Total RNA from the strains AVY30-AVY32 (Table 1) was analyzed by Northern hybridization with *UBR1* (*B*) and *ACT1* (*C*). *Lane a*, RNA from *ubr1* Δ cells (negative control). *Lanes b* and *c*, RNA from wild-type (*UBR1*) cells either in the presence or absence of added Trp (98 μM). *Lanes d* and *e*, same as *lanes b* and *c*, but RNA from *cup9* Δ cells. *D*, Trp-mediated induction of *PTR2* in wild-type and mutant *S. cerevisiae*. Total RNA was isolated from congenic strains of indicated genotypes either before the addition of 98 μM Trp, or 20 min thereafter, or 60 min thereafter, and the relative levels of *PTR2* mRNA were determined by Northern hybridization. *E*, ethidium-stained rRNA (loading controls) in agarose gel before the transfer of RNA to hybridization membrane.

epitope-tagged SSN6 and CUP9 (Fig. 2*B*). Because overexpression of wild-type CUP9 inhibits cell growth, most likely owing to its interactions with DNA (data not shown), we employed a previously constructed nontoxic derivative of CUP9 (3). CUP9 interacts with DNA through its homeodomain motif. Based on the previously studied interactions of other homeodomain proteins with DNA, an Asn \rightarrow Ser substitution at position 265, within the recognition helix of the CUP9 homeodomain, was predicted to strongly reduce the affinity of CUP9 for DNA without causing a significant structural perturbation. This nontoxic CUP9 derivative, tagged at the C terminus with the FLAG epitope, was termed CUP9_{NSF} (3).

Extracts were prepared from *S. cerevisiae* stably expressing both CUP9_{NSF} and SSN6 (SSN6_{myc2}) (the latter C-terminally

tagged with a double *myc* epitope), followed by immunoprecipitation with anti-FLAG antibody, SDS-PAGE, and immunoblotting with anti-*myc* antibody. SSN6_{myc2} was coimmunoprecipitated with CUP9_{NSF} in this assay (Fig. 2*B*, lane *c*). No SSN6_{myc2} was detected in immunoprecipitates of extracts from cells that did not also express CUP9_{NSF} (Fig. 2*B*, lane *b*), and the expression levels of CUP9_{NSF} and SSN6_{myc2} were similar in cells that coexpressed the two proteins (data not shown). Thus, in agreement with the above expectation, these results (Fig. 2*B*) suggested that untagged SSN6 and untagged CUP9, at their (lower) natural expression levels in *S. cerevisiae*, would also form a complex that is analogous to the SSN6/MAT α 2 complex (49), a model that accounts for the observed functional interdependence among CUP9, SSN6, and TUP1 (Figs. 1*B*, 2*A*, and 3*A*). Although we did not examine the immunoprecipitated CUP9_{NSF}-containing complexes for the presence of TUP1, extensive previous work has established that SSN6 and TUP1 form a stable complex both *in vivo* and as purified proteins (Ref. 49, and references therein), indicating that it is the complex of SSN6 and TUP1 that interacts with CUP9, similarly to the previously characterized interaction between SSN6/TUP1 and MAT α 2, another homeodomain repressor (49).

Both CUP9 and UBR1 Are Required for Inducibility of *PTR2* by Amino Acids—When an amino acid, particularly a bulky hydrophobic one such as Leu or Trp, is added to a culture of *S. cerevisiae* growing on a poor nitrogen source such as, for example, allantoin, cells induce their *PTR2* gene and thus increase their capacity for the import of di/tripeptides (32). Effective concentrations of extracellular Leu or Trp can be as low as 1 μM (32). Previous work has shown that an accelerated degradation of CUP9 by the UBR1-dependent N-end rule pathway in response to imported dipeptides with destabilizing N-terminal residues, strongly enhances *PTR2* expression (3). This result led us to consider whether other signals that up-regulate *PTR2* expression, *e.g.* amino acids, may also act through acceleration of CUP9 degradation.

To begin addressing this possibility, we examined the effects of Trp, a strongly inducing amino acid, on *PTR2* mRNA levels in several *S. cerevisiae* strains, AVY32, AVY24, AVY25, AVY30, and AVY31, that were constructed to be completely prototrophic, and in particular did not require extracellular amino acids for viability (Table 1). These strains were grown in allantoin-based media (SHM) containing or lacking Trp. Allantoin is a nonrepressing nitrogen source (32), so the expression of *PTR2* was not influenced by the nitrogen catabolite repression under these conditions. *PTR2* mRNA levels were extremely low in wild-type *UBR1 CUP9* cells grown in medium lacking amino acids, but in the presence of 98 μM (20 $\mu\text{g}/\text{ml}$) Trp the expression of *PTR2* was greatly increased (Fig. 3*A*, lanes *a* and *b*). By contrast, in the absence of CUP9 (in a *cup9* Δ strain), *PTR2* was highly expressed in either the presence or absence of Trp (Fig. 3*A*, lanes *i* and *j*; compare with lanes *a* and *b*). Finally, *PTR2* mRNA was undetectable in a *ubr1* Δ strain grown in either the presence or absence of Trp (Fig. 3*A*, lanes *c* and *d*). Thus, both *UBR1* and *CUP9* are required for inducibility of *PTR2* expression by an amino acid.

Three genes, *SSY1*, *PTR3*, and *SSY5*, are a part of the SPS amino acid-sensing pathway in *S. cerevisiae* (see introduction).

TABLE 1

S. cerevisiae strains used in this study

Strain	Relevant genotype	Reference
JD52	<i>MATa ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801</i>	(56)
JD55	<i>MATa ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 ubr1-Δ1::HIS3</i>	(56)
AVY24	<i>MATα ura3-52 ssy1Δ::myc₃-URA3-myc₃</i>	This study
AVY25	<i>MATα ura3-52 ptr3Δ::myc₃-URA3-myc₃</i>	This study
AVY26	<i>MATα ura3-52 ubr1Δ::HisG</i>	This study
AVY27	<i>MATα ura3-52 ssy1Δ::myc₃</i>	This study
AVY28	<i>MATα ura3-52 ptr3Δ::myc₃</i>	This study
AVY30	<i>MATα leu2-3,112 ubr1Δ::LEU2</i>	(3)
AVY31	<i>MATα leu2-3,112 cup9Δ::LEU2</i>	(3)
AVY32	<i>MATα LEU2</i>	(3)
AVY50	<i>MATa ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 cup9Δ::LEU2</i>	CBY19 in Ref. 1
AVY51	<i>MATa ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 ubr1Δ::HIS3 cup9Δ::LEU2</i>	CBY17 in Ref. 1
AVY60	<i>MATa ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 ssn6Δ::HisG</i>	This study
AVY61	<i>MATa ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 tup1Δ::HisG</i>	This study
AVY62	<i>MATa ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 ubr1Δ::HIS3 ssn6Δ::HisG</i>	This study
AVY63	<i>MATa ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 ubr1Δ::HIS3 tup1Δ::HisG</i>	This study
AVY64	<i>MATa ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 cup9Δ::LEU2 ssn6Δ::HisG</i>	This study
AVY65	<i>MATa ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 cup9Δ::LEU2 tup1Δ::HisG</i>	This study
AVY66	<i>MATa ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 ubr1Δ::HIS3 cup9Δ::LEU2 ssn6Δ::HisG</i>	This study
AVY67	<i>MATa ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 ubr1Δ::HIS3 cup9Δ::LEU2 tup1Δ::HisG</i>	This study
AVY107	<i>MATa ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 ubr1Δ::myc₃</i>	(3)
RJD347	<i>MATα ura3-52</i>	A gift from Dr. R. Deshaies
RJD350	<i>MATα leu2-3,112</i>	A gift from Dr. R. Deshaies
M4272	<i>MATa SUC2 mal gal2 CUP1 ura3 Δ gap1 Δ stp1 Δ stp2</i>	(57)
M4270	<i>MATa SUC2 mal gal2 CUP1 ura3 Δ gap1 Δ stp2</i>	(57)
M4173	<i>MATa SUC2 mal gal2 CUP1 ura3 Δ gap1 Δ stp1</i>	(57)
M4054	<i>MATa SUC2 mal gal2 CUP1 ura3 Δ gap1</i>	(57)
M3750	<i>MATa SUC2 mal gal2 CUP1 ura3</i>	(58)
23344c	<i>MATα, ura3</i>	(59)
JA115	<i>MATα grr1Δ::kanMX2 ura3</i>	(59)

We examined the influence of added Trp on the levels of *PTR2* mRNA in *ssy1Δ* and *ptr3Δ* strains, comparing the consequences of deleting these genes with the effects of deleting either *CUP9* or *UBR1*. The induction of *PTR2* by Trp was dramatically reduced in both *ssy1Δ* and *ptr3Δ* strains (Fig. 3A, lanes e–h). However, a weak Trp-mediated increase in *PTR2* expression was still observed in these strains, in contrast to the complete absence of *PTR2* expression (and of Trp effect) in *ubr1Δ* cells, and in contrast to a high level of *PTR2* mRNA in *cup9Δ* cells (Fig. 3A). Thus the bulk (but apparently not all) of the Trp effect on the expression of *PTR2* requires *SSY1* and *PTR3*.

We next examined whether the general amino acid permease GAP1 (37) was required for Trp-mediated induction of *PTR2* mRNA. Expression of *PTR2* in wild-type cells and a congenic *gap1Δ* strain was measured by Northern analysis as a function of time after the addition of Trp. Wild-type cells exhibited a strong induction of *PTR2* by 20 min in the presence of Trp (Fig. 3, D and E, lanes a–c). Remarkably, the induction of *PTR2* by Trp, while still occurring in the absence of GAP1, was found to be strongly decreased (Fig. 3, D and E, lanes d–f; compare with lanes a–c). To the best of our knowledge, this is the first indication of a major involvement of GAP1 in the amino acid-mediated induction of the *PTR2* peptide transporter. Being an inducer of *PTR2* and other transporter genes via the amino acid sensor *SSY1*, the extracellular Trp exerts its effect without its import across the plasma membrane (see introduction). Therefore the absence of GAP1 as an importer of Trp would not be expected, *a priori*, to influence the *PTR2*-inducing effect of Trp. Moreover, Trp can also be imported by the Trp-specific permease TAP2 (*SCM2*) (51). In sum, the observed decrease (but not elimination) of *PTR2* induction in the absence of *GAP1* (Fig. 3, D and E, lanes a–f) stems from a function of *GAP1* that is

separate from its role as a transporter of amino acids. Indeed, *S. cerevisiae* GAP1 is known to function as an amino acid sensor for activation of protein kinase A targets. The protein kinase A and SCH9 kinases have both overlapping and distinct functions in controlling the adaptation of *S. cerevisiae* to nutrient availability, and GAP1 plays a role at least in the protein kinase A-mediated part of this circuit (Ref. 52, and references therein). Thus, although the mechanistic understanding of involvement of GAP1 in the induction of *PTR2* by Trp remains to be attained, the above finding (Fig. 3, D and E) revealed a new aspect of *PTR2* control and yet another function of GAP1 as a nutritional regulator.

We also measured, in the *gap1* background, the influence of *STP1* and/or *STP2* on the Trp-mediated induction of *PTR2* mRNA. *STP1* and *STP2* are two sequelogenous, conditionally active transcriptional activators that mediate the signaling by the SPS pathway (see introduction) (35, 39, 40, 43). Northern analyses of *PTR2* expression as a function of time after addition of Trp showed that the Trp-mediated induction of *PTR2* was largely retained in a *gap1Δ stp1Δ* strain (which lacked the *STP1* transcriptional activator), in comparison to congenic *gap1Δ* strain, whereas the *STP2* transcriptional activator was essential for *PTR2* induction by Trp: both a *gap1Δ stp2Δ* mutant and a triple mutant *gap1Δ stp1Δ stp2Δ* did not exhibit a significant induction of *PTR2* by Trp, in contrast to parental *gap1Δ* and *gap1Δ stp1Δ* strains (Fig. 3, D and E). Thus, as could be expected from the presence of *STP1/2*-recognized *UAS_{AA}* nucleotide sequence motifs (53) upstream of the *PTR2* gene (data not shown), the induction of *PTR2* proceeds both through its transcriptional up-regulation, in particular by the Trp-activated *STP2* (Fig. 3, D and E), and through a decrease in its repression, owing to the Trp-induced acceleration of degrada-

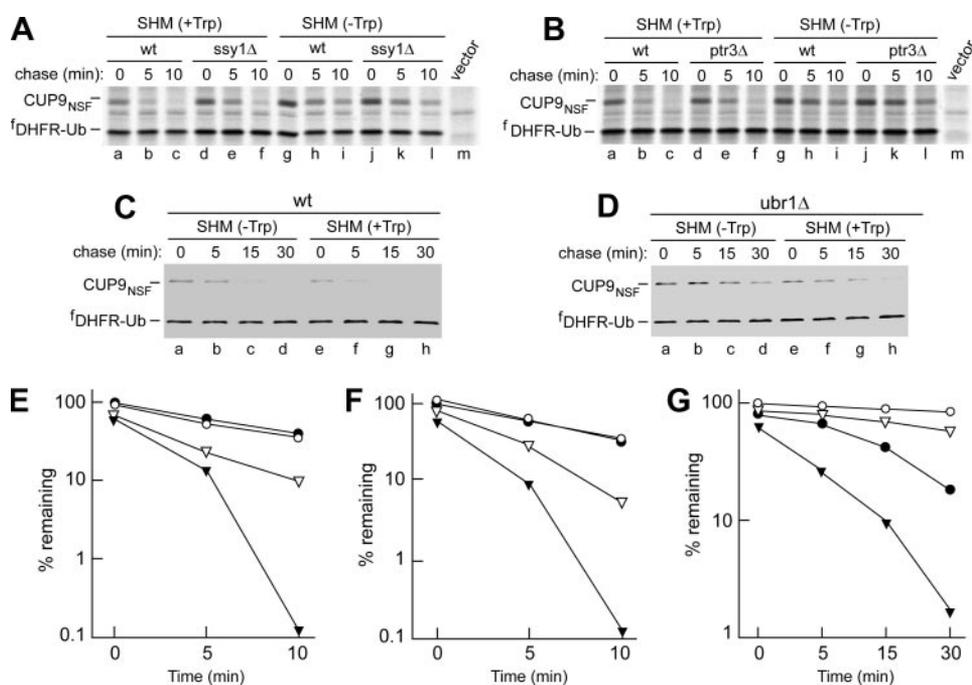


FIGURE 4. The bulk of Trp-accelerated CUP9 degradation requires SSY1, PTR3, and UBR1. Pulse-chase analyses, using the UPR technique and f DHFR-Ub^{K48R} reference protein, of the CUP9 degradation. *S. cerevisiae* of the indicated genotypes were grown in SHM medium containing or lacking 98 μ M Trp (20 μ g/ml). [35 S]Met/Cys labeling for 5 min, followed by chases for 5 and 10 min (A and B) or for 5, 15, and 30 min (C and D), the latter with cycloheximide at 0.2 mg/ml. E and F, PhosphorImager-based quantitation of pulse-chase patterns in A and B, respectively. Closed circles and triangles, wild-type cells, without and with added Trp, respectively. Open circles, either *ssy1* Δ cells (A) or *ptr3* Δ cells (B) in the absence of added Trp. Open triangles, same but with added Trp. G, quantitation of pulse-chase patterns in C and D. Closed circles and triangles, wild-type cells, without and with added Trp, respectively. Open circles and triangles, same but with *ubr1* Δ cells. Data in C, D, and G were from experiments independent of those in A, B, E, and F, and in addition were carried out with cycloheximide. See "Experimental Procedures" for additional details.

tion of the CUP9 repressor by the N-end rule pathway, as described below (Fig. 4).

The SPS Pathway Induces PTR2 Expression through Acceleration of CUP9 Degradation—Some of the above results (Fig. 3A) suggested that the induction of PTR2 transporter by amino acids might occur through an increase in the rate of degradation of CUP9 repressor. A precedent for this possibility was the previously demonstrated acceleration of CUP9 degradation by dipeptides with destabilizing N-terminal residues (3). In the latter case, the bulk of CUP9 degradation, both before and after the addition of dipeptides to the medium, was carried out by the UBR1-dependent N-end rule pathway (3). To determine whether the rate of CUP9 degradation was altered by a PTR2-inducing amino acid such as Trp, we carried out pulse-chase assays with CUP9. These experiments utilized CUP9_{NSF}, the above-described nontoxic, single-residue mutant of CUP9. CUP9_{NSF} was expressed as an f DHFR-Ub^{K48R}-CUP9_{NSF} fusion, where f DHFR is the N-terminally FLAG-tagged mouse dihydrofolate reductase. Deubiquitylating enzymes cotranslationally cleave this fusion at the Ub^{K48R}-CUP9_{NSF} junction, yielding the long-lived reference protein f DHFR-Ub^{K48R} and the test protein CUP9_{NSF} (3). The reference f DHFR-Ub^{K48R} served as a "built-in" internal control for variations in expression levels and immunoprecipitation efficiency. This generally applicable method, called the Ub-protein-reference (UPR) technique (Refs. 12, 47, and references therein), increases the accuracy of pulse-chase assays. The *in vivo* degradation of CUP9_{NSF} was

indistinguishable from that of wild-type CUP9 and was also not altered significantly by the incorporation of this protein into the above (co-translationally processed) UPR-type fusion (3).

We expressed CUP9_{NSF} from a *URA3*-bearing plasmid in *S. cerevisiae* strains that did not require extracellular amino acids for viability (RJD347, AVY26, AVY27, and AVY28; see Table 1). In wild-type (*UBR1*) cells grown in the SHM medium lacking amino acids, CUP9_{NSF} was degraded with $t_{1/2}$ of \sim 10 min (Fig. 4, A, B, E, and F). Remarkably, the addition of Trp (to 98 μ M, or 20 μ g/ml) to the medium resulted in a strong acceleration of CUP9_{NSF} degradation, with $t_{1/2}$ of \sim 2 min between 0 and 5 min of chase, and even $<$ 2 min between 5 and 10 min of chase (Fig. 4, A, B, E, and F). This effect of Trp on the rate of CUP9_{NSF} degradation was reproducible in separate and independent pulse-chase assays, including a set of assays in which cycloheximide, a translation inhibitor, was present during chase (Fig. 4, C and G). (The other assays, in Fig. 4 (A, B, E, and F)

did not involve cycloheximide.) Although in cycloheximide-based assays (Fig. 4, C and G) the CUP9_{NSF} protein was degraded more slowly ($t_{1/2}$ of \sim 20 min and 3–4 min in the absence and presence of Trp, respectively) than in cycloheximide-free assays (Fig. 4, A, B, E, and F), the effect of Trp was comparably strong in both cases (Fig. 4). In addition to increasing the accuracy of pulse-chase assays, the UPR technique, specifically its built-in, long-lived reference protein, makes it possible to determine the relative level of a test protein (measured as the ratio of 35 S in a test versus reference protein) at the beginning of chase, *i.e.* at the end of pulse labeling (12). In Fig. 4 (E–G), 100% was assigned, in each of three panels, to the relative amount of 35 S (relative to the reference protein) of CUP9_{NSF} at time "0" (the end of pulse labeling) in the absence of Trp in the medium. Thus, a value below 100% at time "0" reflects the relative extent of CUP9_{NSF} degradation during the 5-min pulse. In all of pulse-chase assays (Fig. 4, E–G), these initial (time-zero) levels of CUP9_{NSF} were found to correlate, consistently, with Trp-induced changes in the rates of CUP9_{NSF} degradation that were measured during the chase.

The presence of *SSY1* (Fig. 4, A and E) and also, independently, of *PTR3* (Fig. 4, B and F) was found to be strongly but partially required for the Trp-accelerated degradation of CUP9_{NSF}. Specifically, the bulk of enhanced degradation of CUP9_{NSF} upon the addition of Trp was absent in *ssy1* Δ and *ptr3* Δ mutants (Fig. 4, A, B, E, and F). Interestingly, however, the degradation of CUP9_{NSF} was still detectably accelerated by

Induction of Peptide Import

Trp in these mutant strains (Fig. 4, *A, B, E, and F*), in agreement with the weak but still detectable induction, by Trp, of *PTR2* mRNA in *ssy1Δ* and *ptr3Δ* mutants (Fig. 3*A*). As we observed previously as well (3), CUP9_{NSF} was greatly but not completely stabilized in *ubr1Δ* cells (Fig. 4*G* and data not shown). Interestingly, the addition of Trp to *ubr1Δ* cells resulted in a weak but still detectable Trp-induced destabilization of the (now long-lived) CUP9_{NSF}, despite the absence of the N-end rule pathway (Fig. 4*G*, *open circles versus open triangles*). The “residual” (UBR1-independent) instability of CUP9_{NSF} and the residual sensitivity of this degradation to Trp suggest that CUP9 may also be targeted, at a much lower rate, by a UBR1-independent proteolytic pathway. Our attempts to identify a relevant E3 Ub ligase, through the testing of non-UBR1 E3 mutants, have not been successful, thus far (data not shown).

The Levels of UBR1 mRNA Are Not Changed Significantly by a PTR2-inducing Amino Acid—Previous work has shown that the *in vivo* concentration of UBR1 is rate-limiting for degradation of N-end rule substrates by the *S. cerevisiae* N-end rule pathway (reviewed in Ref. 5). To determine whether the Trp-induced acceleration of CUP9 degradation (Fig. 4) was accompanied by an increased level of the UBR1 Ub ligase, we used Northern hybridization to compare the levels of *UBR1* mRNA before and after the addition of Trp. Extracellular Trp did not cause a significant alteration in the levels of *UBR1* mRNA (Fig. 3, *B and C*). Together with findings described in the next section (Fig. 5), these results (Fig. 3, *B and C*) strongly suggested that the observed acceleration of CUP9 degradation in the presence of extracellular Trp (Fig. 4) was not caused by increased levels of UBR1. In addition, the levels of *UBR1* mRNA remained the same or nearly the same in the absence of CUP9 (Fig. 3, *B and C*), indicating that *UBR1* is not a part of the CUP9 regulon.

Degradation of Reporter Proteins Bearing N-degrons Is Not Changed Significantly by a PTR2-inducing Amino Acid—CUP9 lacks an N-degron and is recognized by the UBR1 Ub ligase through a C terminus-proximal degron (see introduction). To determine whether the acceleration of CUP9 degradation by Trp (Fig. 4) was accompanied by increased activity of the N-end rule pathway toward substrates with N-degrons, we employed *X-βgal* reporters. They were produced through cotranslational deubiquitylation of the corresponding Ub-*X-βgal* fusions, where *X* was a variable residue (47, 54). Previous work has shown that the enzymatic activity of βgal in extracts from cells that expressed an *X-βgal* reporter protein was a reliable measure of the reporter's metabolic stability *in vivo* (27). Of the four *X-βgal* reporters in Fig. 5, Met-βgal and Ala-βgal had stabilizing N-terminal residues, whereas His-βgal and Tyr-βgal had, respectively, a type-1 and type-2 primary destabilizing N-terminal residues. As would be expected from the UBR1-dependent degradation of His-βgal and Tyr-βgal (5), their levels in wild-type (*UBR1*) cells were much lower than the levels of long-lived Met-βgal and Ala-βgal under the same conditions (Fig. 5). We found that the addition of Trp, which led to a strong acceleration of the UBR1-dependent degradation of CUP9 (Fig. 4), did not result in significant alterations of the relative steady-state levels of His-βgal and Tyr-βgal reporters, in comparison to those of Met-βgal and Ala-βgal (Fig. 5). These findings were independently confirmed by carrying out pulse-chase assays

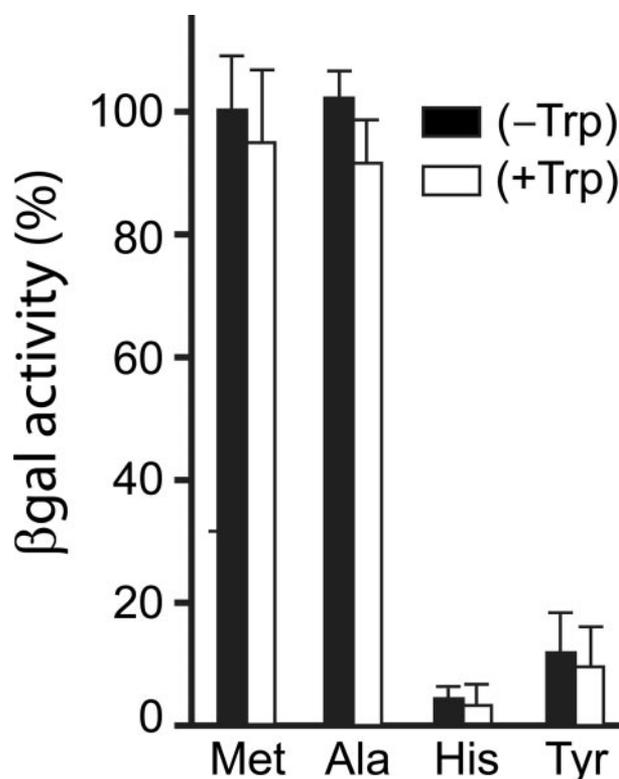


FIGURE 5. Relative metabolic stabilities of *X-βgal* test proteins in the absence or presence of added tryptophan. *In vivo* degradation of *X-βgal* proteins (*X* = Met, Ala, His, or Tyr) was assessed by comparing their steady-state levels in wild-type *S. cerevisiae* in the absence or presence of added Trp. In contrast to CUP9, which is targeted for UBR1-dependent degradation through its internal, C terminus-proximal degron, the short-lived His-βgal and Tyr-βgal (bearing, respectively, a type-1 and type-2 destabilizing N-terminal residue) are targeted by UBR1 via their respective N-degrons.

with *X-βgal* reporter proteins (data not shown). Thus the activity of the N-end rule pathway toward substrates with N-degrons did not change significantly upon the addition of Trp.

Concluding Remarks—Extracellular Trp does not increase the activity of the N-end rule pathway toward its N-degron-bearing substrates, whereas it does accelerate degradation of the CUP9 repressor by the same pathway (Figs. 4 and 5), thereby making possible the induction of the *PTR2* peptide transporter. This dichotomy constrains possible interpretations of our findings. Yet another constraint stems from the fact that the bulk of the Trp-induced acceleration of CUP9 degradation by the N-end rule pathway requires the amino acid-sensing SPS pathway (Figs. 3*A* and 6). CUP9 is targeted for degradation via its internal degron, through its interaction with a third substrate-binding site of the UBR1 E3 Ub ligase, which functions as the UBR1-RAD6 holoenzyme (see introduction). The autoinhibited CUP9-binding site of UBR1 can be activated, allosterically, by dipeptides with destabilizing N-terminal residues that interact with the other two (type-1 and type-2) binding sites of UBR1 (2, 3). Conformational equilibria, in the *in vivo* ensembles that contain UBR1, would determine a fraction of UBR1 that is active toward CUP9. We suggest that such equilibria can be shifted toward CUP9-targeting conformations of UBR1 not only in response to UBR1-binding di/tripeptides, as demonstrated previously (2, 3), but also through other routes, for example UBR1 phosphorylation. Other studies have shown

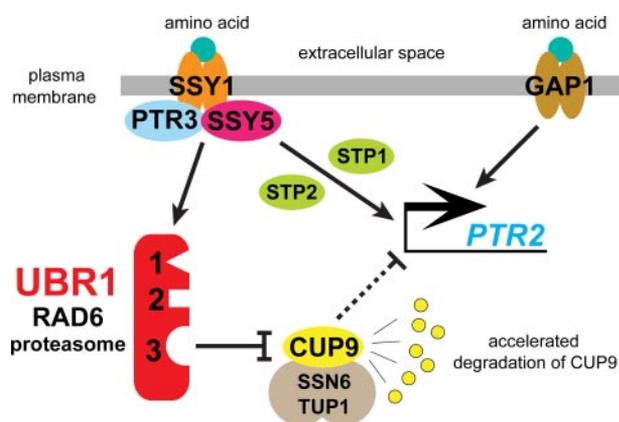


FIGURE 6. The amino acid-sensing SPS pathway and its connections with the PTR2-CUP9-UBR1 circuit, including the amino acid-mediated acceleration of degradation of CUP9, a transcriptional repressor of the PTR2 peptide transporter. See the main text for details, including the evidence for involvement of a GAP1-mediated pathway.

that signaling by the SSY1 sensor of amino acids that results in the PTR3/SSY5-dependent (proteolytic) activation of the latent transcription factors STP1 and STP2 is mediated, at least in part, by an increased phosphorylation of PTR3, in reactions mediated by the SSY1-associated YCK1/YCK2 kinases (35, 40, 55). In sum, a parsimonious, testable model that can account for our findings (Figs. 1–5) and is also consistent with other evidence is that the Trp-activated signaling by the SPS system (Fig. 6) may increase the rate of CUP9 degradation by the N-end rule pathway through a phosphorylation of the UBR1 Ub ligase. This phosphorylation may be mediated, at least in part, by the previously described SSY1-associated kinases that mediate the SSY1-induced phosphorylation of PTR3 (35, 40, 55). (As shown in Figs. 3 and 4, both SSY1 and PTR3 are required for the bulk of Trp-induced acceleration of CUP9 degradation.)

Our recent findings⁶ indicate that UBR1 is a multiply phosphorylated protein. They also indicate that UBR1 phosphorylation involves a set of kinases that includes YCK1/YCK2, and that the resulting modifications of UBR1 regulate its functions.⁶ Work is underway to dissect UBR1 phosphorylation *in vivo* and to determine whether specific kinds of phosphorylated UBR1 underlie the Trp-induced acceleration of degradation of the CUP9 repressor (Fig. 4). Work is also underway to identify one or more putative E3 Ub ligases that appears to mediate the residual degradation of CUP9 in *ubr1Δ* cells, which lack the N-end rule pathway. Yet another aspect of these circuits that remains to be understood is a mechanism that underlies our finding (Fig. 3, *D* and *E*) that the general amino acid permease GAP1 contributes to the induction of PTR2 by Trp. Given recent studies of GAP1 as an amino acid sensor (Ref. 52 and references therein), its Trp-mediated effect on PTR2 is likely to involve a phosphorylation-based pathway. A better understanding of spatiotemporal aspects of CUP9 regulation would also be important. Because steady-state levels of (short-lived) CUP9 in wild-type cells are very low, and because overexpression of wild-type CUP9 is toxic, this and previous studies (2, 3) utilized CUP9_{NSF}, a missense mutant that does not bind to cognate DNA sites that CUP9 normally recognizes. The Trp-induced acceleration of CUP9_{NSF} degradation (Fig. 4) implies that *in vivo* interactions of CUP9 with DNA do not play a sig-

nificant role in the Trp effect. Nevertheless, it would be important to determine whether wild-type CUP9 can be targeted for degradation by the N-end rule pathway directly at sites of its association with DNA *in vivo*, whether a modification of CUP9, e.g. its phosphorylation, might also be involved in the Trp-induced, UBR1-dependent acceleration of CUP9 degradation, and whether this degradation takes place in the context of CUP9-containing complexes such as CUP9-SSN6-TUP1. Some of the other transcriptional repressors, such as MAT α 2, are metabolically stabilized by their association with SSN6-TUP1 (50).

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