Regulation of the Gts1p Level by the Ubiquitination System to Maintain Metabolic Oscillations in the Continuous Culture of Yeast*

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Yeast cells exhibit sustained ultradian oscillations of energy metabolism in coupling with cell cycle and stress resistance oscillations in continuous culture. We have reported that the rhythmic expression of Gts1p is important for the maintenance of ultradian rhythms. Structurally, Gts1p contains sequence motifs similar to N-degron and the ubiquitin association domain, raising the possibility that the Gts1p level is regulated by degradation via ubiquitination. When the lysine residue at the putative ubiquitination site of the N-degron was substituted with arginine, both the protein level and half-life of mutant Gts1p increased. During continuous culture, the protein level of the mutant Gts1p was elevated and did not fluctuate, leading to the disappearance of metabolic oscillation within a day. Furthermore, using three Gts1ps containing mutations in the ubiquitin association domain, we showed that the lower the binding activity of the mutant Gts1ps for polyubiquitin, the higher the protein level in vitro. Expression of the mutant Gts1ps in the continuous culture resulted in an increase in Gts1p and early loss of the oscillation. Therefore, Gts1p is degraded through conjugation with ubiquitin, and the UBA domain promoted the degradation of ubiquitinated Gts1p, causing a fluctuation in protein level, which is required for the maintenance of metabolic oscillations.

In an open system using a bioreactor, yeast cells exhibit sustained ultradian oscillations of energy metabolism in a continuous (chemostat) culture under aerobic and glucose-limited conditions (1–4). The energy metabolism pathway has been proven to be an autogenous oscillator under extreme nonequilibrium energy conditions according to the theory of “dissipative structures” established by Prigogine and others (5–7). In brief, the metabolic pathway oscillates autonomously under the primary control of phosphofructokinase, transferring energy from glucose to NADH, which acts as the feed-forward activator, and then from NADH to ATP, which acts as the feedback inhibitor. After ATP as an inhibitor is consumed as fuel for various ATPases, glucose again begins to enter the glycolytic pathway. The oscillations are detectable as a periodic change in the factors involved in energy metabolism such as dissolved oxygen (DO) levels, CO₂ production, glucose and ethanol concentrations, and amounts of storage carbohydrates. DO oscillation is due to the periodic change between respiratory and respiro-fermentative phases in which oxygen demands are relatively high and low, respectively. DO oscillations arise spontaneously in concert with cell division and are dependent on a high cell density (~5 x 10⁶ cells/ml), regulating the cell density throughout the oscillation (8). We have reported that cellular responses to various stress conditions, such as heat, oxidative agents, and cytotoxic compounds, are regulated in concert with metabolic oscillation (8) and that the coupling is disrupted by inactivation of the GTS1 gene (9). Furthermore, we suggested that the rhythmic expression of Gts1p is more important than protein level for the maintenance of ultradian rhythms, since the constitutive expression of GTS1 under the control of the TPI promoter resulted in the disappearance of ultradian rhythms (9). More recently, we reported that, when GTS1 was expressed under the control of a short (183-bp) promoter in the GTS1-disrupted mutant, the amplitude of Gts1p fluctuations was restricted, leading to the attenuation of the metabolic oscillation and to the uncoupling of stress-resistance oscillations (10). Thus, we suggested that, in order for stress resistance oscillations to occur, a full fluctuation in the level of Gts1p is required. Furthermore, we found that the mRNA level of GTS1 fluctuated with a broad peak at the respiro-fermentative phase, which is the opposite of the Gts1p fluctuation (10), suggesting that GTS1 expression was regulated in an oscillatory manner at the transcriptional level. However, it is likely that the oscillatory expression of GTS1 is not sufficient to cause a fluctuation in the protein level, since the protein needs to be degraded for any subsequent increase to occur and/or for regulation of the protein level.

In this paper, we examined whether the fluctuation in the level of Gts1p is controlled by the ubiquitin-proteasome system, since we found that Gts1p contains a putative N-degron (11) and ubiquitin association (UBA) domain (12). Using site-directed mutagenesis at the sequence motifs, we examined the effects of mutations on the fluctuation in Gts1p expression and the oscillations in energy metabolism during continuous culture. The results suggested that the fluctuation in the level of Gts1p is regulated by the ubiquitination system in order to maintain the oscillations of energy metabolism, in combination with the oscillatory GTS1 expression at the transcriptional level.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—The haploid strain S288C (MATa ma gal1/2 SUC2) of Saccharomyces cerevisiae was used. FL589 (see †) was a gift from Dr. J. Nittis (Harvard University). In the batch cultures, cells were incubated at 30 °C in a synthetic medium containing...
sisting of a yeast nitrogen base without amino acids (Difco) containing 2% glucose with the required amino acids and bases.

**Conditions for Continuous Culture**—The cells were cultured at 30 °C, pH 5.5, in a synthetic medium containing 1% glucose as defined elsewhere (2) using an improved bench top fermenter, MLD-6C (Marubishi Bioengineering, Tokyo), at a constant volume of 500 ml (8). Continuous cultures were performed at a dilution rate of 0.1 h⁻¹ with aeration at 1 liter/h and stirring at 420 rpm. The periodic change in respiratory-fermentative metabolism was monitored by measuring the level of DO with an oxygen electrode.

**Construction of Mutant GTS1**—To obtain the wild type GTS1, a 2.30-kilobase pair XhoI-SpeI fragment containing both GTS1 and a 1.0-kilobase pair upstream region was obtained by PCR directed against genomic DNA using oligonucleotides GTS1-Xho and GTS3-DS' (Table I and Fig. 1A) as 5'- and 3'-primers, respectively, and inserted into the multicloning site of the centromere-based vector pRSA103, which is derived from pRS413 (Stratagene, La Jolla, CA) containing AUR1 from pAUR112 (Takara, Tokyo) in place of HIS3 (9). The XbaI site in the multicloning site of the recombinant plasmid was removed by deletion of the Snacl-Spee fragment to obtain pRSA-GTS1.

For construction of GTS1[K17R] encoding Gts1p[K17R] containing a substitution at position 17 (arginine for lysine), a PCR-amplified fragment obtained using GTS1-Nhe and K17R-2 as 5'- and 3'-primers, respectively, was digested with XhoI and XbaI and inserted into the cognate sites of pRSA-GTS1 to obtain pRSA-GTS1[K17R] (Table I). The mutation in pRSA-GTS1[K17R] was confirmed by sequencing the GTS1 site in the recombinant plasmid and incorporated into the GTS1-disrupted strain gts1Δ (8, 9).

To construct GTS1[TGF] encoding a substitution in MGF between amino acids 205 and 207 of Gts1p, a fragment amplified using TGF and GTS3-Sal as 5'- and 3'-primers (Table I) was ligated with a fragment obtained using K17R-1 and TGF-Spe (Table I) as primers, after digestion with ApIII. The PCR product amplified on the ligated fragment using GTS-Xho and GTS-Spe (Table I) as 5'- and 3'-primers, respectively, was digested with XhoI and XbaI and inserted into the cognate sites of pRSA-GTS1 to obtain pRSA-GTS1[TGF] (Table I). The mutation in pRSA-GTS1[TGF] was confirmed by sequencing the GTS1 site in the recombinant plasmid and incorporated into the GTS1-disrupted strain gts1Δ (8, 9).

**Determination of mRNA Levels by Northern Blotting**—The cells were cultured at 30 °C, pH 5.5, in a synthetic medium containing 1% glucose as defined elsewhere (2) using an improved bench top fermenter, MLD-6C (Marubishi Bioengineering, Tokyo), at a constant volume of 500 ml (8). Continuous cultures were performed at a dilution rate of 0.1 h⁻¹ with aeration at 1 liter/h and stirring at 420 rpm. The periodic change in respiratory-fermentative metabolism was monitored by measuring the level of DO with an oxygen electrode.

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For construction of GTS1[K17R] encoding Gts1p[K17R] containing a substitution at position 17 (arginine for lysine), a PCR-amplified fragment obtained using GTS1-Nhe and K17R-2 as 5'- and 3'-primer (Table I), respectively, was ligated with a fragment obtained using K17R-1 and TGF-Spe (Table I) as primers, after digestion with ApIII. The PCR product amplified on the ligated fragment using GTS-Xho and GTS-Spe (Table I) as 5'- and 3'-primers, respectively, was digested with XhoI and XbaI and inserted into the cognate sites of pRSA-GTS1 to obtain pRSA-GTS1[K17R] (Table I). The mutation in pRSA-GTS1[K17R] was confirmed by sequencing the GTS1 site in the recombinant plasmid and incorporated into the GTS1-disrupted strain gts1Δ (8, 9).

To construct GTS1[TGF] encoding a substitution in MGF between amino acids 205 and 207 of Gts1p, a fragment amplified using TGF and GTS3-Sal as 5'- and 3'-primer (Table I), respectively, was ligated with a fragment amplified using GTS5-RI and GTS3-Sal as primers, after digestion with BsrI and SalI, respectively. The EcoRI-SalI segment from the ligated fragment was inserted into the cognate sites of pYX222 (Takara), and the BglII-XbaI fragment obtained from the recombinant plasmid was inserted into the cognate sites of pYX222 to give the recombinant plasmid pYX222 to give the recombinant plasmid pYX222. After confirmation of the mutations in the mutant GTS1 genes in the recombinant plasmids, the transformation of gts1Δ cells proceeded.

**Treatment with a Proteasome Inhibitor and Western Blotting**—FL599 (ise1) was cultured using the A500 value reached 1.0 and mixed with MG132 at a final concentration of 50 μM. After incubation for a specific period, cells were harvested, and the protein levels of Gts1p and actin in the cell lysates were determined by Western blotting as described previously (9). The relative protein level of Gts1p to actin was determined with the lumino-image analyzer (LAS1000; Fuji Film, Tokyo).

**Determination of mRNA Levels by Northern Blotting**—Northern blotting of GTS1 and GTS1[K17R] mRNAs was performed using as probes digoxigenin-labeled PCR products amplified with the DIG labeling kit.
The Yeast Two-hybrid System

The two-hybrid analysis was performed using a Matchmaker two-hybrid system (CLONTECH, Palo Alto, CA) as described previously (14). The open reading frame of UBI4 encoding five ubiquitin proteins in a head-to-tail arrangement (15) was amplified on genomic DNA using UBI-5/H11032RI and UBI-3/H11032Sal (Table I) as 5'- and 3'-primer, respectively, and the EcoRI-SalI fragment from the PCR product was inserted into the cognate sites of pGAD424 in-frame with the gene encoding the activation domain of GAL4. On the other hand, the open reading frames of wild type and mutant GTS1 genes were amplified in the recombinant plasmid pRSA103 using GTS-5/H11032RI and GTS-3/H11032Sal (Table I) as 5'- and 3'-primer, respectively. The EcoRI-SalI fragments obtained from the PCR products were inserted into the cognate sites of pGTB9 in-frame with the gene encoding the DNA-binding domain of GAL4. The interactions between the prey and bait hybrid proteins were determined for the activation of the lacZ reporter gene by measuring the β-galactosidase activity of the cells using the liquid culture assay (14).

RESULTS

Ubiquitin-related Amino Acid Sequences in Gts1p and the Effect of a Proteasome Inhibitor on the Gts1p Level—Gts1p contains two sequences homologous to ubiquitin-related motifs, N-degron and the UBA domain (Fig. 1). If the first methionine residue is removed from Gts1p in vivo, arginine, one of the destabilizing residues, is located at the N terminus and the ubiquitin-binding residue lysine occupies position 17. In addition, basic residues are present at positions 3 and 10, which have been reported to increase the efficiency of N-degron (16). Thus, the N-terminal sequence of Gts1p almost completely fits the consensus sequence of N-degron if the first methionine residue is removed. On the other hand, the UBA domain con-
sists of about 40 amino acid residues comprising three α-helices linked by two loops (12, 17). The putative UBA domain in Gts1p has 80% homology to the consensus sequence including three well conserved amino acid residues (Gly-206, Leu-217, and Ala-227) (Fig. 1B). The presence of these sequences would suggest that Gts1p is degraded by the ubiquitin-proteasome system. To test this possibility, the effect of the proteasome-specific inhibitor MG132 on the degradation of Gts1p was examined using FL599 (ise1) cells having enhanced permeability to the drug (13). Western blotting analysis after the treatment with MG132 in vivo showed that the Gts1p level had increased by more than 4-fold 4 h after the treatment with the drug (Fig. 2), suggesting the involvement of proteasomes in the degradation of Gts1p.

**Association of Gts1p with Ubiquitin**—To investigate whether Gts1p binds ubiquitin in vivo, we first tried to detect ubiquitin-Gts1p conjugates in the wild type cells but could not detect any signals by Western blotting using anti-ubiquitin antibody in the immunoprecipitate with anti-Gts1p antibody (data not shown). Then we constructed a mutant overexpressing HA-tagged Gts1p (HA-Gts1p), named TMpHA-GTS1, since we thought that Gts1p-ubiquitin conjugates might be minor components. Western blot analysis of the cell lysate from TMpHA-GTS1 using anti-Gts1p showed that there are several signals in addition to the one corresponding to HA-Gts1p at 45 kDa (Fig. 3A). Although the additional signals could not be found in the

![Graph](image)

**Fig. 5. Effect of mutation at the putative ubiquitin-ligation site of N-degron of Gts1p on its own degradation rate.** Time courses of the protein levels of Gts1p (A) and Gts1p(K17R) (B) relative to actin were determined by Western blotting after the treatment with cycloheximide. Cells were treated with 50 µg/ml cycloheximide and incubated for specified periods. Half-lives of Gts1p and Gts1p(K17R) were estimated to be 3.5 and about 12 h, respectively.

**Fig. 6. Effect of mutation at the putative ubiquitin-ligation site of N-degron of Gts1p on the oscillation of the energy metabolism.** A, the DO oscillation in gts1Δ expressing the wild type Gts1p with the centromeric recombinant plasmid carrying pRSA-GTS1 with its upstream region of about 1.0 kilobase pair. B, the DO oscillation in gts1Δ cells expressing Gts1p(K17R) with the plasmid pRSA-GTS1[K17R]. The horizontal bar indicates the time when cell samples for C were collected. C, the fluctuation of the Gts1p level during the DO oscillation in gts1Δ cells expressing Gts1p(K17R). The open square in the column marked WT indicates the relative Gts1p level at the maximum of the fluctuation during the DO oscillation in the wild type cell as a control. The broken line indicates a schematic diagram showing the fluctuation of the Gts1p level in A superimposed over the OD oscillation as a comparison (the patterns of Western blotting were shown in Ref. 9). These are representative patterns of three independent experiments.
Western blot of cell lysate from the wild type cell, we thought that they are possibly some modified forms of Gts1p, since none of them was found in the cell lysate from gts1Δ (Fig. 3A).

To detect Gts1p-ubiquitin conjugates in TmpHA-GTS1, the cell lysate was immunoprecipitated with anti-HA antibody, and Gts1p-ubiquitin conjugates were detected in the immunoprecipitate by Western blotting using anti-ubiquitin antibody (Fig. 3B). A major signal suggestive of Gts1p-ubiquitin conjugates had a molecular mass of 55 kDa, which corresponds approximately to that of HA-tagged Gts1p (45.6 kDa) conjugated with one molecule of ubiquitin (8.5 kDa) (Fig. 3B, right lane). Since there is no band at the position corresponding to the 55-kDa signal in the Western blot of the original lysate obtained using anti-Gts1p antibody (Fig. 3B, left lane), the 55-kDa conjugate is thought to be a minor component in the Gts1p population. In addition, none of the signals found in the Western blot of the lysate (Fig. 3, left lane) turned out to be a ubiquitin conjugate. At present, we do not know the nature of these signals. It should be pointed out that there are some minor bands stained like a smear in the high molecular weight region above the major band. Thus, the results suggested that Gts1p is conjugated with ubiquitin in a predominantly monoubiquitinated form.

**Effect of Mutation at the Putative Ubiquitin Ligation Site of N-degron of Gts1p on the Metabolic Oscillation**—To examine whether the N-degron of Gts1p is involved in its degradation, a mutant Gts1p(K17R) containing arginine at position 17 instead of lysine was expressed in the Gts1p (data not shown). However, when the whole cell lysate was expressed in the yeast two-hybrid system (18). We investigated whether the N-degron of Gts1p is involved in the degradation of protein necessary for the fluctuation at the protein level and support the notion that periodicity change in the Gts1p level is necessary for the persistent DO oscillation during the continuous culture.

**The Binding Activity of the UBA Domain of Gts1p for Ubiquitin**—The UBA domain has been identified in various classes of proteins including those in the ubiquitin-proteasome system (12). The function of the domain has not been elucidated although it was reported that the UBA domains of Rad23p and Ddi1p, DNA-damage-inducible proteins, bind to ubiquitin non-covalently in the yeast two-hybrid system (18). We investigated whether the UBA domain of Gts1p can associate with ubiquitin using the yeast two-hybrid system. When the monoubiquitin gene, which was amplified by polymerase chain reaction from UBI4 encoding five ubiquitin genes in tandem (15), was used as a bait in the system, Gts1p as prey did not bind to the protein (data not shown). However, when the whole UBI4 gene was used as bait, Gts1p did bind to the polyubiquitin protein, although the binding activity as determined using β-galactosidase was about half that of Gts1p proteins (14) (Table III). To further determine the binding activity of Gts1p to ubiquitin via the UBA domain, we introduced site-directed mutations in the amino acid triplet MGF between positions 205 and 207, since G-206 is the one of the most conserved residues among UBA domains (12) (Fig. 1). We constructed four mutant Gts1ps containing TGF, MAF, MGQ, and MAQ in place of MGF, named Gts1p(TGF), and so on. However, since gts1Δ expressing Gts1p(MAF) grew very slowly, it could not be used in further experiments. We do not know the reason why gts1Δ expressing Gts1p(MAQ), which contains another mutation at G-207, grew fairly well, but it should be pointed out that MAF is more hydrophobic than the wild type MGF, whereas MAQ retains some hydrophilicity similar to that of MGF. Of the three mutant Gts1ps tested, Gts1p(MAQ) was reduced most in terms of the activity to bind ubiquitin followed by that of

| Strains                  | Gts1p level (Peak/through = amplitude)a | DO oscillation | Durationb |
|-------------------------|-----------------------------------------|----------------|------------|
| Wild type               | 100/35 = 2.86                           | 40.4±2.2       | 132        |
| gts1Δ transformed with  |                                         |                |            |
| pRSA-GTS1               | 95/33 = 3.03                            | 28.4±2.9       | 117        |
| pRSA-GTS1[K17R]         | 178/134 = 1.32                          |                |            |
| pRSA-GTS1[MAQ]          | 175–213 (nof)                           |                |            |
| pRSA-GTS1[TGF]          | 100–102 (no)                            |                |            |
| pRSA-GTS1[TGQ]          | 105/74 = 1.42                           | 35.6±2.4       | 60         |

a Relative level of Gts1p to actin at the peak and trough of the fluctuations. The Gts1p level in each strain was normalized taking the peak level of the wild type cells as 100.

b Average and S.D. of the amplitude of waves (n > 20) of the DO oscillation estimated by subtracting the DO concentration (%) of the trough from that of the peak of the next wave.

c Average duration (h) of the DO oscillation.

d no, no significant fluctuation in the protein level.

Though the amplitude of DO oscillation was about 30% lower than in the wild type cells (Table II). The Gts1p level oscillated at a level nearly identical to that of the wild type cells (data not shown) as reported previously (9). In contrast, the oscillation in gts1Δ cells expressing Gts1p(K17R) ceased within a day (Fig. 6B). The Gts1p(K17R) level was twice the peak value of Gts1p in the wild type cells during the transient DO oscillation (Fig. 6B). The fluctuation in the level of Gts1p(K17R) was severely restricted (Fig. 6C) compared with that of the wild type Gts1p expressed in either the wild type or gts1Δ cells transformed with GTS1 (9) (Table II). In addition, the amplitude of the cell cycle oscillation also decreased. These results suggest that the N-degron of Gts1p is involved in the degradation of protein necessary for the fluctuation at the protein level and support the notion that periodicity change in the Gts1p level is necessary for the persistent DO oscillation during the continuous culture.
Gts1p(TGF) (Table III). Since the binding activity of Gts1p(Maq) to ubiquitin did not change as much as that of the wild type, Gly-206 is the most important residue for binding in the three mutant Gts1ps, which is consistent with the fact that Gly-206 is well conserved among UBA sequences. Thus, it is likely that the UBA domain of Gts1p can associate with ubiquitin, as shown in a previous report (18).

Effect of Mutations in the UBA Domain of Gts1p on Its Degradation—To examine whether the UBA domain is involved in the degradation of Gts1p itself, the protein levels of the mutant Gts1ps were measured (Fig. 7). Among the three mutant Gts1ps, the protein level of Gts1p(Maq) was increased the most, followed by that of Gts1p(TGF). This was the reverse of the case for the binding activity for polyubiquitin (Table III), showing that the lower the binding activity for polyubiquitin, the lower the degradation capacity of the protein itself.

Effect of Mutations in the UBA Domain of Gts1p on the Metabolic Oscillation—When Gts1p(Maq), whose activity to bind ubiquitin was most affected among the three, was expressed in gts1Δ/h9004 in continuous culture, no oscillation in the concentration of DO was seen (Fig. 8). The Gts1p level was about twice the peak value in the wild type cells and seemed to gradually increase (Fig. 8 and Table II). On the other hand, Gly-206 is well conserved among UBA sequences. Thus, it is likely that the UBA domain of Gts1p can associate with ubiquitin, as shown in a previous report (18).

**TABLE III**

| Plasmids | Prey |
|----------|------|
| pGAD424b | pGBT-GTS1 |
| pGAD-GTS1 | pGBT-GTS1 |
| pGAD-UBI4 | pGBT-GTS1 |
| pGAD-UBI4 | pGBT-GTS1(TGF) |
| pGAD-UBI4 | pGBT-GTS1[MAQ] |
| pGAD-UBI4 | pGBT-GTS1[MGQ] |

| Activity (n = 3) | β-Galactosidase Percentage of control |
|-----------------|-------------------------------------|
| 0.70 ± 0.08     | 6 |
| 11.4 ± 1.66     | 100 |
| 5.14 ± 0.35     | 45 |
| 3.72 ± 0.23     | 33 |
| 2.20 ± 0.18     | 19 |
| 4.78 ± 0.25     | 42 |

*These are representative data of three independent experiments assayed in triplicate.

*b Vector alone.
gts1Δ cells expressing Gts1p(TGF) whose activity to bind ubiquitin was intermediate among the three showed a transient DO oscillation, which disappeared within a day (Fig. 9A and Table II). The Gts1p(TGF) level during this transient oscillation did not fluctuate and remained equivalent to the peak value of Gts1p (Fig. 9B). In contrast, gts1Δ cells expressing Gts1p(TGQ) exhibited a metabolic oscillation with a high amplitude similar to that of the wild type cell (Table II). However, the oscillation did not last as long (~3 days) (data not shown), probably because the amplitude of the Gts1p(TGF) level was significantly restricted, although the protein level was similar to that of the wild type cell (Table II). These results suggested that the UBA domain of Gts1p is involved in the degradation of the protein to regulate the fluctuation in the protein level, which is in turn required to maintain the oscillation of energy metabolism.

**DISCUSSION**

In this report, we suggested that Gts1p was degraded via the ubiquitin-proteasome system through ubiquitination at K-17, although it is not clear whether the first methionine, one of the stabilizing residues according to the N-end rule (11), is removed. In fact, we previously sequenced the N-terminal portion of Gts1p isolated from yeast overexpressing the protein under the control of the TPI promoter in a multicopy vector and found that this protein, if not all proteins, contained the first methionine. However, the result does not eliminate the possibility that the overexpressed Gts1p partly evades excision of the first methionine. The results of the present study showing that Gts1p had a half-life of 3.5 h in the wild type cells after cycloheximide treatment suggested that Gts1p has a destabilizing residue at the N terminus, taking into consideration that inhibition of protein synthesis with cycloheximide generally promotes the stabilization of proteins and mRNAs. Furthermore, the K17R mutation resulted in an increase in the level of Gts1p and a longer half-life, suggesting ubiquitination at Lys-17. In addition, we detected a Gts1p-ubiquitin conjugate in cell lysate, although it was of a monoubiquitinated form. This result is in contrast to other reports (19, 20), where overexpressed Myc and x-ray-induced p53 formed multiquitin conjugates in a ladder-like fashion. Monoubiquitination has been reported to target membrane-bound proteins for endocytosis, leading to eventual degradation in vacuoles (21, 22). However, it is unlikely that Gts1p is degraded in vacuoles, since the protein is located in the supernatant of the cell lysate and the degradation is inhibited by the proteasome inhibitor MG132. It is possible that the Gts1p associated with multiquitin chains undergoes rapid degradation.

We showed that the UBA domain of Gts1p is involved in the interaction with ubiquitin using the two-hybrid assay and that the domain promoted the degradation of Gts1p itself. The result of the assay is consistent with the report by Bertolaet al. (18) in which the UBA domains of Rad23p and Ddi1p bind ubiquitin noncovalently. The result showing that Gts1p did not bind to the ubiquitin monomer in the two-hybrid system is in close agreement with a recent report (23) that indicates UBA domain-containing proteins like Rad23 and Mud1 bind more than 300 times more tightly to polyubiquitin than to monoubiquitin. The binding of the polyubiquitin of the three mutant Gts1ps at the UBA domain in vitro decreased in parallel with the capacity for degradation of Gts1p in vivo. Gts1p(MAQ) containing a Gly to Ala substitution at position 206, which most affected the association with polyubiquitin, was most deficient in the degradation of Gts1p. The Gly residue is one of the most conserved in the UBA domain and is located in the loop linking α-helices 1 and 2 and is known to comprise a part of the hydrophobic surface to which the ubiquitin molecule is thought to bind (18, 24). The result suggested that the UBA domain is involved in the degradation of multiquitin- nated Gts1p in an autoregulated (autocatalytic) manner, since the expression of Gts1p(MAQ) which contains an intact N-degron resulted in the complete loss of the fluctuation of Gts1p level in the continuous culture. This may be a reason why multiquitin- nated Gts1p was not detected in cell lysate by Western blotting. However, since this domain is contained in many proteins without N-degron and reportedly interacts with proteins other than ubiquitin (24), the possibility remains that it has as yet unknown functions in addition to the degradation of Gts1p itself.

In this report, we presented evidence that the degradation of Gts1p by the ubiquitin-proteasome system is necessary for a rhythmic expression of the protein. It now remains to be elucidated when and how the ubiquitination system is activated during metabolic oscillation in a continuous culture. Since the ubiquitin-proteasome system requires a large amount of ATP, it is assumed that the system is activated in the respiratory phase when much more ATP is produced in mitochondria than in the respiro-fermentative phase when glycolysis is predominant. Consistently, the decrease of Gts1p begins in the middle of the respiratory phase (9, 10). However, since the expression of GTS1 is regulated in an oscillatory manner at the transcription level (10), the regulation at the rhythmic expression of Gts1p may be much more complicated than anticipated here.

It would be interesting to note that the ubiquitin-proteasome system is involved in the regulation of biological rhythm in higher organisms. The three clock-related PAS proteins from Arabidopsis contain the F-box characteristic of proteins that direct ubiquitin-mediated degradation (25, 26), and in Drosophila a clock-related protein called timeless (TIM) is known to be degraded by the ubiquitin-proteasome system in response to light (27). These and our results suggested the involvement of the ubiquitin-proteasome system in the regulation of biological rhythms.

In this report, we showed, for the first time, that the ubiquitin-proteasome system is involved in the regulation of the ultradian oscillation of the energy metabolism by means of degrading Gts1p in yeast. These results are very important and suggest for further study on the molecular mechanism of how ultradian rhythms in yeast are regulated. Furthermore, since the energy metabolism pathway has been proven to be an autogenous oscillator in dissipative structures including all living organisms, these data will contribute to studies on the biological rhythms in other organisms.

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