Gln3p is a nitrogen catabolite repression-sensitive GATA-type transcription factor. Its nuclear accumulation was recently shown to be under the control of TOR signaling. Gln3p normally resides in the cytoplasm. When cells are starved from nitrogen nutrients or treated with rapamycin, however, Gln3p becomes translocated into the nucleus, thereby activating the expression of genes involved in nitrogen utilization and transport. To identify other genes under the control of Gln3p, we searched for the Gln3p-binding GATAA motifs within 500 base pairs of the promoter sequences upstream of the yeast open reading frames in the Saccharomyces Genome Database. APG14, a gene essential for autophagy, was found to have the most GATAA motifs. We show that nitrogen starvation or rapamycin treatment rapidly causes a more than 20-fold induction of APG14. The expression of APG14 is dependent on Gln3p; deletion of Gln3p severely reduced its induction by rapamycin, whereas depletion of Ure2p caused its constitutive expression. However, overexpression of APG14 led to only a slight increase in autophagy in nitrogen-rich medium. Therefore, these results define a signaling cascade leading to the expression of APG14 in response to the availability of nitrogen nutrients and suggest that the regulated expression of APG14 contributes to but is not sufficient for the control of autophagy.

In response to nutrient starvation conditions, particularly nitrogen starvation, autophagy acts as an emergency measure to generate an internal supply of nutrients. It may also serve to reduce energy-consuming cellular activities, such as protein synthesis, by nonselectively delivering cytosolic materials to the lysosome (higher eukaryotes) or vacuole (yeast) for degradation (reviewed in Ref. 1). Failure to undergo autophagy severely compromises the viability of cells during starvation. In addition, autophagy is also involved in selectively removing aged organelles such as mitochondria under normal growth conditions in higher eukaryotes. During autophagy, a portion of the cytoplasm is sequestered by a double- or multilayered membrane structure referred to as the autophagosome or autophagy body. Autophagosomes are then transported to and fused with the lysosome or vacuole, resulting in the eventual degradation of cytoplasmic materials by the lysosomal or vacuolar proteases (reviewed in Ref. 1).

Genetic approaches have been undertaken to identify the players involved in autophagy. A number of autophagy (APG or AUF) genes have been identified that are required for autophagy (2, 3). In a separate genetic screen for genes involved in cytoplasm to vacuole targeting, it was found that there is an overlap of many common components between the cytoplasm to vacuole targeting and autophagy pathways (4). Emerging biochemical evidence indicates that the autophagy genes are involved in various steps in the formation and delivery of autophagosomes. For example, Apg6p/Apg8p/Apg13p/Apg14p form a peripheral membrane-associated complex. No autophagosomes were observed in the vacuoles of apg6Δ or apg14Δ cells (5). Thus the Apg6p-Apg14p complex appears to be involved in the initial step(s) of autophagy. Apg6p has a dual role in both autophagy and vacuolar protein sorting, whereas Apg14p appears to be designated solely for autophagy. Overexpression of Apg14p can suppress the defect of apg6Δ in autophagy but not vacuolar protein sorting (5). Apg8p/Aut7p/Cvt5p forms a complex with Apg4p/Aut2p, a microtubule-binding protein (6, 7). Mutations in APG4 or APG8 cause the accumulation of autophagosomes in the cytoplasm, suggesting that the Apg4p-Apg8 complex is responsible for the delivery of autophagosomes to the vacuole. Once autophagosomes are fused with the lysosome/vacuole, vacuolar acidification by the vacuolar H+-ATPase is required for the activity of vacuolar proteases (e.g. Cps1p and Prb1p) to break down the cytosolic materials into small molecules such as amino acids, resulting in the eventual disappearance of autophagosomes (reviewed in Refs. 1 and 8).

Autophagy has long been morphologically associated with a variety of human disorders. Elevated autophagy is seen in several human degenerative diseases such as Alzheimer’s and Parkinson’s (9, 10). In contrast, autophagy is significantly reduced in many cancer cells (reviewed in Ref. 11). Beclin 1 is a Bcl-2-interacting protein and the human homolog of Apg6p. It complements the autophagic but not the vacuolar protein-sorting function of Apg6p in yeast (12). beclin 1 is frequently deleted in sporadic breast and ovarian cancers, and its expression level is significantly decreased in human breast epithelial carcinoma cell lines and tissues (12, 13). Overexpression of beclin 1 leads to elevated autophagy, reduced proliferation of cancer cells, inhibition of in vitro clonigencity, and tumorigenesis in nude mice (12). Taken together, these results indicate that the Apg6p-Apg14p complex plays an important role in the regulation of autophagy.

Rapamycin is a macrocyclic immunosuppressive antibiotic that inhibits the growth of eukaryotic cells. The targets of rapamycin (TOR) genes, TOR1 and TOR2, were initially iso-
regulated for their dominant mutations causing rapamycin resistance (14–16). It was later shown that FKBP12-rapamycin directly binds to TOR and that their dominant rapamycin-resistant mutants at a conserved serine residue, Ser-1972 in Tor1p or Ser-1975 in Tor2p, disrupt the binding of FKBP12-rapamycin (17–19). The binding of FKBP12-rapamycin to TOR is mediated by a small 12-kDa domain called the FKBP12-rapamycin-binding (FRB) domain (18, 20). The three-dimen-
sional structure of the FKBP12-rapamycin-FRB complex shows that the conserved serine residue is located in the hydrophobic rapamycin-binding pocket of FRB (21). A substitution of the conserved serine with a bulky amino acid prevents the binding of rapamycin to the hydrophobic pocket. TOR proteins are highly conserved evolutionarily and belong to the ataxia telan-
giectasia mutated (ATM)-related kinase family (reviewed re-
cently in refs. 22 and 23). The yeast TOR proteins have protein serine and threonine kinase activities (24–26). TOR has emerged as an important regulator of nutrient-mediated sig-
aling. Rapamycin treatment causes phenotypes typical of
starvation responses, including severely reduced protein syn-
thesis (27, 28), glycogen accumulation (27), and autophagy (29). Rapamycin treatment or mutations in both TOR1 and TOR2 leads to autophagy in the absence of starvation (29). In addi-
tion, rapamycin treatment also causes elevated autophagy in
mammalian cells, indicating that the ability of TOR to regulate autophagy is conserved.

Gln3p is a nitrogen catabolite repression (NCR)-sensitive GATA-type transcription factor. It is required for the expres-
sion of genes involved in the transport and utilization of nitro-
gen compounds (reviewed in Ref. 30). It normally resides in the
cytoplasm. The Gln3p pathway has been recently shown to be under the control of TOR (31–34). When cells are growing in poor nitrogen sources or under starvation, Gln3p rapidly accumu-
lates in the nucleus (31, 32), thereby activating the trans-
cription of NCR-sensitive genes such as general amino acid permease (GAP1) and glutamine synthetase (GLN1) (31–34). TOR directly binds to and phosphorylates Gln3p, leading to the cytoplasmic retention of Gln3p (31). The inhibition of TOR by rapamycin causes the dephosphorylation and nuclear accumu-
lation of Gln3p (31, 32). Ure2p is a pre-prion protein genetically identified as an inhibitor of Gln3p (reviewed in Ref. 30). Ure2p binds to Gln3p and appears to inhibit the dephosphorylation step of Gln3p (31). The depletion of Ure2p also leads to the
dephosphorylation and nuclear accumulation of Gln3p (31, 32). TOR signaling is also implicated in the regulation of several other transcription factors. It binds to Gat1p, Gzf3p, Dal80p, Dal81p, and Dal82p (31). Rapamycin treatment also causes nuclear accumulation of Gat1p, Msn2p, and Msn4p (32).

In this study, we performed a DNA pattern search for the potential target genes of Gln3p. We found that APG14 is among three genes with the largest number of Gln3p-binding motifs (GATAA). We further showed that the inhibition of TOR by rapamycin or nitrogen starvation leads to the rapid induction of APG14 in a Gln3p-dependent manner. Interestingly, the overexpression of APG14 itself caused a slightly increased level of autophagy. These observations defined a signaling cas-
cade leading to the expression of APG14 in response to nitrogen starvation. They showed that regulated expression of APG14 may contribute to but is not sufficient for the control of autophagy.

**Experimental Procedures**

**Strains and Plasmids**—The strains and plasmids were FM391 (MATa hisΔ1, leu2Δ200, met15Δ0, uro3Δ0) (a gift from M. Johnston) and Szy159 (FM391, ure2Δ:KanMX) (33), Szy415 (FM391, pho8Δ60), and Szy426 (Szy159, pho8Δ60). The PHOS in the Szy strains was replaced with pho8Δ60 by inserting the plasmid pTN9 (a gift from Dr. Y. Oh-
sumi) into the PHOS locus and excised out as previously described (38). The APG14 open reading frame was cloned into pAUD6 plasmid with a Muc epitope tag in front of the starting codon ATG.

**Northern Blot Analysis**—Exponential wild type and mutant yeast cultures were treated with 200 nm rapamycin or drug vehicle. Aliquots of yeast cultures were withdrawn at different times. For nitrogen and carbon starvation, logarithmic cells in the synthetic complete medium were switched to synthetic complete minus nitrogen sources or carbon sources, and samples were withdrawn at different times. Total yeast RNAs were prepared using the freezing phenol extraction method (39). Twenty μg of total yeast RNA samples were separated on denaturing agarose gels, transferred onto nylon filters, hybridized to 32P-labeled DNA probes, and detected by a phosphorimaging device (Bio-Rad).

**Western Blot Analysis**—Log-phase yeast cells were harvested and lysed with glass beads in disruption buffer (50 mM Tris- HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40) plus a mixture of protease inhibitors (Roche Molecular Biochemicals) by vortexing. For Western blot analysis, 10 μg of protein samples were used for gel electrophoresis and detected by ECL (Amersham Pharmacia Biotech) with monoclonal antibody 9E10.

**Microscopy**—Exponential wild type and mutant yeast cells were treated with rapamycin (or a drug carrier [methanol]) in the presence or absence of 1 mM phenylmethylsulfonyl fluoride. Aliquots of yeast cultures were withdrawn and analyzed for the appearance of autophago-
somes by a Zeiss Axiosplan 2 microscope equipped with a Nomarski objective lens and a SPOT digital camera system (Diagnostic Instruments, Inc.). Consistent with a previous finding (36), we found that phenylmethylsulfonyl fluoride treatment alone did not cause autophagy but significantly enlarged the size of autophagosomes, which is conven-
ient for the analysis of autophagy.

**Alkaline Phosphatase Assay**—The assay basically followed that de-
scribed by Noda et al. (38). Yeast cells were harvested in phosphate buffer (PB, 250 mM Tris, pH 9.0, 10 mM MgSO4, and 10 μM ZnSO4), disrupted with glass beads, and centrifuged at 10,000 × g for 20 min. The protein concentrations of the yeast extracts were measured by the Bradford assay (Bio-Rad). Fifty μg of protein were incubated with 25 mM α-naphthyl phosphate (Sigma) at 30 °C for 30 min. The reactions were stopped by the addition of 0.5 ml of 2× glycine-NaOH (pH 11.0) and measured by the fluorescence intensity of emissions at 472 nm after excitation at 345 nm.

**Results**

Recent gene expression profiling analysis has revealed that the inhibition of TOR by rapamycin leads to a rapid global change in the expression of NCR-sensitive genes as well as other diverse yeast genes (31, 33, 34). Therefore, it is possible that Gln3p plays a broader role in the control of gene expres-
sion beyond NCR-sensitive genes. To identify new target genes for Gln3p, we carried out a pattern search for the Gln3p-binding motif GATAA in 500 base pairs of the 5′-untranslated region (UTR) of yeast open reading frames. This approach was inspired by the observation that Gln3p-dependent genes often contain multiple GATAA motifs in their 5′-UTR (reviewed in Ref. 30). MEP2, APG14, and YOR142w are found to each contain eight GATAA motifs, the largest number in the entire yeast genome (The APG14 5′-UTR is shown in Fig. 1a). MEP2 encodes for ammonia transporter previously shown to be under the control of Gln3p (31). In addition, two other Gln3p-dependent, NCR-sensitive genes, AMID2 (amidase) and DAL5 (allan-
toate permease), contain seven GATAA motifs. Therefore, multiple GATAA motifs in the 5′-UTR appear to be a good indicator for potential Gln3p-dependent genes. TOR has been linked to the control of autophagy and regulation of Gln3p. APG8 was also recently shown to be inducible by nitrogen starvation (7, 35) and rapamycin (Ref. 33 and Footnote 2). Thus APG14 is a likely Gln3p target gene and therefore is controlled by TOR signaling. To confirm this, we performed Northern blot analy-

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1 The abbreviations used are: FRB, FKBP12-rapamycin-binding; NCR, nitrogen catabolite repression; UTR, untranslated region; ADH, alcohol dehydrogenase; YPD, yeast extract-peptone-dextrose.

2 T.-F. Chan, P. G. Bertram, W. Ai, and X. F. Zheng, unpublished observations.
caused elevated expression of APG14 in cells carrying the wild type TOR1 but not the TOR1\textsuperscript{RR} cells (Fig. 1c). Thus the inhibition of TOR by rapamycin is directly responsible for the induction of APG14. Because phosphorylation of Gln3p by TOR is important for the cytoplasmic retention and inhibition of Gln3p, we also examined whether an intact TOR kinase domain is required for the repression of APG14. In this experiment, wild type yeast cells carrying a plasmid-borne TOR1(S1972I,D2294E) were used (Fig. 1c). TOR1(S1972I,D2294E) contains two independent point mutations: the S1972I mutation in the FKBP12-rapamycin-binding (FRB) domain that prevents the binding of FKBP12-rapamycin and the D2294E mutation located at a critical site of the kinase catalytic domain that completely abolishes the kinase activity of Tor1p (18). Even though FKBP12-rapamycin is unable to bind to Tor1p(S1972I,D2294E), Tor1p(S1972I,D2294E) failed to repress the induction of APG14 in the presence of rapamycin (Fig. 1c). Thus an intact kinase domain of TOR is required for the normal repression of APG14.

TOR has been implicated in the control of a large number of transcription factors, including Gln3p, Gat1p, Gzf3p, Dal80p, Dal81p, Dal82p (31), Msn2p, and Msn4p (32). Because Gln3p, Dal80p, and Gat1p are GATA-type transcription factors, we asked whether they are involved in the expression of APG14. We found that the deletion of GLN3 severely reduced APG14 expression in the presence of rapamycin (Fig. 2). A residual expression of APG14 in the absence of Gln3p appears to be mediated by Gat1p (data not shown). Ure2p is the inhibitor of Gln3p in the presence of preferred nitrogen sources. Rapamycin or the deletion of URE2 leads to the nuclear accumulation of Gln3p and constitutive expression of several Gln3p-dependent genes such as GAPI and GLN1 (31, 32). Similar to GAT1 and GLN1, the ure2Δ mutation led to the constitutive expression of APG14 in the absence of rapamycin (Fig. 2). Rapamycin treatment did not further increase APG14 expression in the ure2Δ strain (Fig. 2). Taken together, Gln3p is primarily responsible for mediating the induction of APG14 by rapamycin.

The starvation of either nitrogen or carbon sources causes autophagy (reviewed in Ref. 1). We next examined the effect of deprivation of nitrogen nutrients on APG14. Similar to rapamycin treatment, we found that nitrogen starvation rapidly induced the expression of APG14 (Fig. 3). However, glucose starvation had no discernible change in the transcript levels of APG14 (data not shown). These findings are consistent with our observation that glucose starvation did not significantly cause autophagy in the strains examined in this study (data not shown). Thus nitrogen, but not carbon, starvation causes elevated expression of APG14.

The accumulation of autophagosomes in the yeast vacuole is a hallmark for cells undergoing autophagy and has been used as a convenient microscopic measurement of autophagy (36, 37). Because rapamycin activates both Gln3p and autophagy,
we investigated whether the activation of Gln3p is sufficient for autophagy. In this experiment, we chose to use the ure2Δ strain that contains the active form of Gln3p. Similar to rapamycin treatment, the deletion of URE2 caused the accumulation of a large number of autophagosomes in nearly all the cells in a mid-log phase culture in YPD (Fig. 4, a and b). To confirm the above observation, we also performed a Pho8Δ60p-based alkaline phosphatase assay (38). The alkaline phosphatase Pho8p is normally localized on the vacuolar membrane. Pho8Δ60p is instead localized in the cytoplasm because of the truncation of its transmembrane region and can only be delivered into the vacuole by autophagy. Once in the vacuole, Pho8Δ60p can be processed by vacuolar proteases and becomes the active form that can be detected by the alkaline phosphatase activity assay (38). As previously reported (29), alkaline phosphatase activity increased 2.9-fold by rapamycin treatment in the wild type yeast (Fig. 4c). The ure2Δ mutation also caused an ~2.3-fold increase in alkaline phosphatase activity over that in the wild type strain (Fig. 4c). Therefore, the activation of Gln3p by URE2 deletion appears to cause autophagy at a level comparable with that by rapamycin.

Apg14p forms a heterodimer with Apg6p. A recent study shows that overexpression of beclin 1, the human homolog of Apg6p, caused elevated autophagy in human breast carcinoma cells (12). However, the level of Apg6p, caused elevated autophagy in human breast carcinoma cells (12). Therefore, the induction of APG14 expression is highly inducible by rapamycin, we examined whether the overexpression of APG14 has any effect on autophagy. We engineered Apg14p under the control of the alcohol dehydrogenase (ADH) promoter on a 2 μm plasmid. We found the presence of high steady state levels of APG14 mRNA (Fig. 5a) and protein (Fig. 5b) in cells carrying the ADH-APG14 but not in the vector control plasmid. The APG14 mRNA level is at least 5- to 10-fold more than the endogenous APG14 mRNA induced by rapamycin (data not shown). In yeast cells expressing Apg14p, however, there was only a slight increase in the autophagy level over the control cells (Fig. 5, c and d). This is in contrast to the rapamycin-treated wild type cells or the untreated ure2Δ cells (Fig. 4). Therefore, the induction of APG14 does not appear to be sufficient for autophagy. Other factor(s) under the control of the Gln3p pathway must also be involved.

**DISCUSSION**

Autophagy is a cellular process that nonselectively delivers cytosolic materials to the lysosome (higher eukaryotes) or vacuole (yeast) for degradation (reviewed in Ref. 1). It can generate an internal supply of nutrients and is essential for cell viability during starvation. Because autophagy nonselectively degrades many cytosolic materials such as ribosomes, it is not desirable under normal growth conditions. Not surprisingly, autophagy is a highly regulated process; it occurs at very low levels when cells are growing in nutrient-rich medium but at high levels during starvation. Cancer cells appear to have developed a strategy to gain growth advantage by deleting beclin1/APG6 and decreasing their autophagy levels (12). Therefore, it is important to understand the control of APG genes and the regulatory circuit of autophagy. Biochemical and genetic evidence indicates that the APG genes play critical roles in the formation and transport of autophagosomes (reviewed in Ref. 1). Mutations in these genes prevent yeast from forming autophagosomes or delivering them to the vacuole during starvation. A recent study establishes that TOR plays an important role in the control of autophagy (29). Rapamycin treatment or compromise in both TOR1 and TOR2 genes leads to high levels of autophagy in the absence of starvation. In this study, we discovered that the 500-base pair upstream region of APG14 is highly abundant in Gln3p-binding sites (Fig. 1a). Further investigation shows that APG14 is indeed highly inducible by nitrogen starvation or rapamycin treatment (Figs. 1b and 3). The expression of APG14 is primarily under the control of Gln3p; the deletion of GLN3 severely reduced APG14 expression by rapamycin, whereas activation of Gln3p by the ure2Δ mutation led to the constitutive expression of APG14. Our results also show that the inhibition of TOR kinase activity is necessary for the induction of APG14.

Apg6p and Apg14p form a peripheral membrane-bound complex (5). Earlier studies indicate that this protein complex is critical for autophagy. The deletion of either APG6 or APG14 completely inhibits autophagy. In contrast, the overexpression of beclin 1, the human homolog of Apg6p, promotes autophagy.
in human breast carcinoma cells (12). Apg6p has a dual function in both autophagy and vacuolar sorting in yeast. In contrast, Apg14p is specific for autophagy (5). Unlike mammalian cells, we found that the transcript level of Apg6p remained unchanged during starvation or rapamycin treatment. In contrast, Apg14 is highly induced under these conditions. Because the overexpression of beclin 1 leads to a significant increase in autophagy, we examined the effect of Apg14 overexpression on autophagy in yeast. Unlike the unc2Δ mutation or rapamycin treatment, which leads to autophagy in virtually all cells (Fig. 4) (29), we found that Apg14 overexpression only slightly increased autophagy (Fig. 5, c and d). Thus additional factor(s) appear to contribute to the onset of autophagy. Apg8p may be one such protein. The abundance of Apg8 increased approximately 8-fold following the starvation of nitrogen nutrients (7, 35). The expression of Apg8 also increased similarly as a result of rapamycin treatment (data not shown) (33). We also found that Gln3p is responsible for the expression of Apg8 (data not shown). Apg8p interacts with Apg4p, a microtubule-associated protein (6). The apg4Δ and apg8Δ mutations cause the accumulation of autophagosomes in the cytoplasm, suggesting that Apg4p or Apg8p is responsible for the delivery of the autophagosomes to the vacuole (6, 7). Therefore, autophagy may require the simultaneous induction of several components of the autophagy apparatus.

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