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Tor, a Phosphatidylinositol Kinase Homologue, Controls Autophagy in Yeast*

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Autophagy is a bulk protein degradation process that is induced by starvation. The control mechanism for induction of autophagy is not well understood. We found that Tor, a phosphatidylinositol kinase homologue, is involved in the control of autophagy in the yeast, *Saccharomyces cerevisiae*. When rapamycin, an inhibitor of Tor function, is added, autophagy is induced even in cells growing in nutrient-rich medium. A temperature-sensitive *tor* mutant also leads to induction of autophagy at a nonpermissive temperature. These results indicate that Tor negatively regulates the induction of autophagy. Tor is the first molecule that is identified as a pivotal player in the starvation-signaling pathway of autophagy. Furthermore, we found that a high concentration of cAMP is inhibitory for induction of autophagy. *APG* gene products are involved in autophagy induced by starvation. Autophagy was not induced in *apg* mutants in the presence of rapamycin, indicating that the site of action of Tor is upstream of those of Apg proteins. In nutrient-rich medium, Apg proteins are involved also in the transport of aminopeptidase I from the cytosol to the vacuole. Tor may act to switch Apg function between autophagy and transport of aminopeptidase I.

When nutrients are depleted from the environment, mammalian as well as yeast cells begin to degrade their own cytosol and organelles. This bulk protein degradation is mediated mainly by autophagy, the process in which cytoplasmic components are delivered to the lysosome (1). In the process of autophagy, a portion of cytoplasm is sequestered by a double or multi-layered membrane structure, referred to as the autophagosome (1). The autophagosome fuses with endosomal or lysosomal membranes, resulting eventually in degradation of cytoplasmic materials (1, 2).

To initiate this dynamic reorganization of intracellular membranes, as occurs during formation of autophagosome, cells are expected to have a regulatory mechanism of autophagy. Although the overall mechanism remains to be elucidated, some factors have been reported to be involved in this control in mammalian cells. For example, formation of autophagosomes is inhibited by amino acids (3, 4), by insulin (5), by cell swelling (6), and by cAMP (7). Blommaart *et al.* reported that the phosphorylation of ribosomal protein S6 and autophagy are controlled by the same signal transduction pathway (8). S6 phosphorylation is mediated by the serine/threonine kinase p70S6k (9).

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In the yeast *Saccharomyces cerevisiae*, autophagy is induced by starvation (10). The sequence of events in yeast is essentially the same as that of mammalian cells: formation of an autophagosome is followed by fusion of the autophagosome to the vacuole, which is equivalent to a lysosome in mammalian cells (11). The inner membrane structures of the autophagosome are delivered to the vacuole as the autophagic bodies (11). The autophagic bodies are destroyed depending on proteinase B activity in the vacuole (10). However, the induction mechanism of autophagy in yeast is not well understood.

Nutrient starvation not only induces autophagy but also arrests cells at early G_1 phase. Tor, a phosphatidylinositol kinase-related kinase, is known to be involved in the signaling pathway from nutrient starvation to G_1 arrest in yeast (12–15). The immunosuppressive drug rapamycin is effective in arresting yeast at early G_1 phase (12). The effect of rapamycin is mediated by FKBP, and it is the FKBP-rapamycin complex that binds to Tor (16, 17). There are two Tors in yeast, and inactivation of both Tors leads to G_1 arrest (15). In addition to inhibiting G_1 progression, Tor2p has another essential function that appears to be involved in the organization of the actin cytoskelton (13, 14, 18). Tor1p does not have this function, which is not inhibited by rapamycin (19). To investigate the control mechanism of autophagy in yeast, we determined the effects of inactivating Tor and of a high concentration of cAMP.

EXPERIMENTAL PROCEDURES

Yeast Strains—TN125 cells were constructed from wild-type strain YPH499 (*MAT***a** *ura3–52 lys2–801 ade2–101 trp1-Δ1 his3-Δ200 leu2-Δ1*) obtained from the Yeast Genetic Stock Center (Berkeley), and TN126 cells were constructed from HM128–9A (*MAT***a** *lys2–801 leu2-Δ1 trp1-Δ1 ura3–52 ade2–101 his3-Δ200 pde2–28 pde1–28 cyr1–28*) (20), a gift from Dr. Mitsuzawa at the National Institute of Genetics (Mishima, Japan). The *PHO8* gene in each strain was replaced with *pho8Δ60* by inserting the plasmid pTN9 into *PHO8* locus and excised out as described previously (21). TN127 cells were constructed by disrupting the *FKB1* gene of TN125 cells using the plasmid fkb1URA3, a gift from Dr. Tanida (Tonen Corporation). TN128 cells were made by replacing the *PHO8* gene with *pho8Δ60* as above in SH221 (*leu2–3, 112 ura3–52 rme1 trp1 his3 HMLa ade2Δ tor1::HIS3–3 tor2::ADE2–3*/YCp*lac111::tor2–21*^{ts}), a gift from Dr. M. Hall (University of Basel). MT55–4-3 (*MAT***a** *apg4 leu2 ura3*), MT37–4-2 (*MAT***a** *apg5 leu2 ura3*), and MT91–4-2 (*MAT***a** *apg10 ura3*) were constructed in our laboratory (22).

Yeast Culture—Yeast cells were grown in YPD (1% yeast extract, 2% polypeptone, 2% glucose). For the nitrogen starvation, the cells in log phase were washed with water and suspended in SD(−N) medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate (Difco) and 2% glucose). Rapamycin (Sigma) was added at a final concentration of 0.2 μg/ml using a stock solution of 20 μg/ml in 90% ethanol and 10% Triton X-100. Hydroxyurea (Sigma) was added to 0.2 M, and nocodazole (Sigma) was added to 10 μg/ml using stock solution of 10 mg/ml in dimethyl sulfoxide. cAMP (Sigma) was added at a final concentration of 1 mM.

Flow Cytometry—Cultures (0.3 ml) of yeast in YPD medium (A_{600} = 0.5) were added to 0.7 ml of ethanol. The samples were incubated overnight at −20 °C, washed, then resuspended, and sonicated for 30 s in 200 mM Tris-Cl (pH 7.5), 50 mM EDTA. The samples were then

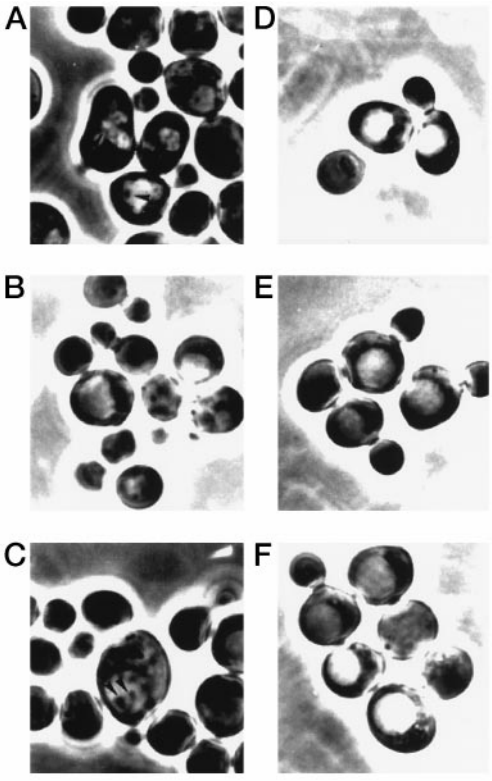

FIG. 1. **Induction of autophagy by rapamycin.** *A–C*, wild-type strain (TN125); *D*, *apg4* strain (MT55–4-3); *E*, *apg5* strain (MT37–4-2); *F*, *apg10* strain (MT91–4-2). *A*, cell in SD(−N) medium with 1 mM phenylmethylsulfonyl fluoride; *B–F*, cells in YPD medium containing 1 mM phenylmethylsulfonyl fluoride. *C–F*, 0.2 μg/ml of rapamycin was added to the medium. After incubation for 3 h at 30 °C, the photographs were taken. The autophagic bodies are indicated by the *arrowheads*.

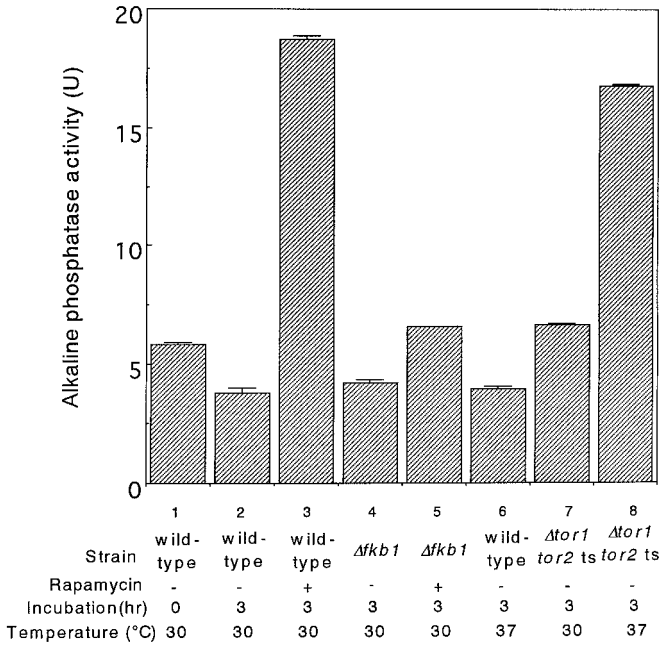

FIG. 2. **The effect of Tor inactivation on autophagy monitored by biochemical assay.** Each cell culture in YPD medium was incubated at 30 or 37 °C in the absence or the presence of 0.2 μg/ml of rapamycin. After 0 or 3 h, the cells were harvested and subjected to alkaline phosphatase assay to measure autophagic activity. Protein concentration was determined by the BCA method. Representative results of three experiments are presented. Wild type (TN125); Δ*fkb1* (TN127); Δ*tor1 tor2*^{ts} (TN128).

incubated with RNase (0.2 mg/ml) for 4 h at 37 °C. DNA was stained by 0.2 mg/ml propiodium iodide in 200 mM Tris-Cl (pH 7.5), 50 mM EDTA for 1 h at 4 °C. 10,000 events were analyzed for DNA content using Coulter Epics XL.

Alkaline Phosphatase Assay—The assay method was basically that of Nothwehr *et al*. (23). Cells (1–2 × 10^{7}) were harvested and washed with water. The cells were then suspended in 0.2 ml of assay buffer (250 mM Tris-HCl (pH 9.0), 10 mM $MgSO_4$, 10 μM $ZnSO_4$) and disrupted by vortexing with glass beads. After centrifugation, 50 μl of the supernatant was added to 0.5 ml of assay buffer and 50 μl of 55 mM α-naphtylphosphate to start the reaction. After incubation at 30 min for 20 °C, 0.5 ml of 2 M glycine-NaOH (pH 11.0) was added to stop the reaction. Fluorescence intensity of emission at 472 nm after excitation at 345 nm was measured. One unit is defined as the activity to release 1 μmol α-naphtol/min/mg protein. Protein concentration was determined by the BCA method (Pierce) or the Bradford method (24).

RESULTS

Autophagy in yeast is induced under starvation conditions (10) and can be followed as an accumulation of autophagic bodies in the vacuole when vacuolar degradation is inhibited by inactivation of proteinase B (Fig. 1*A*). In nutrient-rich YPD medium, autophagy usually does not occur (Fig. 1*B*). However, when rapamycin was added to cells growing in YPD, it was found that autophagic bodies accumulate in the vacuole, indicating that autophagy was induced (Fig. 1*C*). This morphological observation was confirmed by use of the biochemical assay for autophagy developed previously (21). The assay is based on the fact that the vacuolar membrane protein alkaline phosphatase, Pho8p, becomes localized in the cytoplasm if its transmembrane region is truncated (21). When autophagy occurs, this truncated Pho8p, Pho8Δ60p, is delivered to the vacuole where it becomes active as a result of processing by vacuolar proteases (21). Therefore, the measurement of phosphatase activity provides a simple assay for autophagic activity (21). When rapamycin was added to YPD culture of cells harboring *pho8Δ60*, alkaline phosphatase activity increased, implying that autophagy had been induced (Fig. 2, *lane 3*).

Rapamycin is known to bind to the FK506 binding protein, FKBP, to effect cell cycle arrest in yeast (12). We investigated whether FKBP is involved in this induction of autophagy by rapamycin. Disruption of *FKB1*, the gene for FKBP, resulted in the failure of rapamycin to induce autophagy (Fig. 2, compare *lane 3* and *5*). This result indicates that the action of rapamycin in inducing autophagy is mediated by FKBP.

The FKBP-rapamycin complex inhibits Tor function (16, 17). Inactivation of the two *TOR* genes leads to G_1 arrest (15). The *Pho8Δ60* gene was introduced into cells in which both *TOR1* and *TOR2* were deleted and harbored temperature-sensitive allele of *TOR2* in a plasmid. This strain grows at 30 °C but not at 37 °C (15).[1] Alkaline phosphatase activity was increased when *tor2*^{ts} cells harboring *Pho8Δ60* were incubated in YPD medium for 3 h at 37 °C, whereas it was not increased at 30 °C in the same cells or in the wild-type *TOR* cells at 37 °C (Fig. 2, *lanes 6–8*). Based on these results, we concluded that inactivation of Tor function is sufficient for induction of autophagy.

It has been reported that treatment with rapamycin or inactivation of Tors leads to cell arrest at early G_1 phase (15). This raises the possibility that autophagy is induced when the cell cycle is arrested at any phase. To investigate this possibility, we treated cells with hydroxyurea to arrest the cell cycle at S phase or with nocodazole to arrest the cell cycle at M phase. After 3 h of treatment with these reagents, flow cytometry analysis confirmed that most cells were arrested at S or M phase. After treatment with hydroxyurea, 83% of the are with 1 N DNA as compared with 56% in control cells. After treatment with nocodazole, 76% of the cells are with 2 N DNA as compared

[1] M. Hall, personal communication.

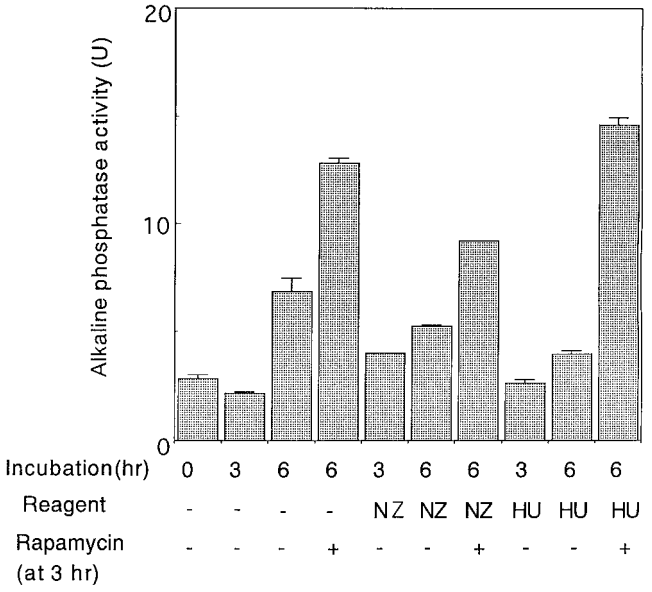

FIG. 3. **Relationship between autophagy and cell cycle.** Hydroxyurea or nocodazole was added to the cell cultures of wild-type strain TN125 cells in YPD medium. After 3 h, rapamycin was added to the half of each cell culture and incubated further for 3 h. At the time indicated, the cells were harvested and subjected to alkaline phosphatase assay to measure autophagic activity. Representative results of three experiments are presented.

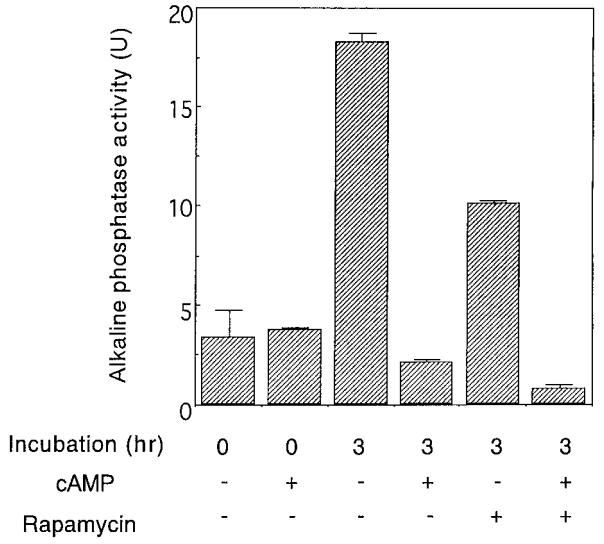

FIG. 4. **The effect of cAMP on autophagy.** Triple mutant strain (TN126) in cAMP pathway was transferred to starvation medium, SD(−N) in the absence or the presence of 1 mM cAMP and/or 0.2 μg/ml of rapamycin at 0 h. After 3 h at 30 °C, the cells were harvested and subjected to alkaline phosphatase assay to measure autophagic activity. Protein concentration is determined by the BCA method. Representative results of three experiments are presented.

with 44% in control cells. However, in those cell cycle arrested cells, autophagy was not induced (Fig. 3). Moreover, when rapamycin was added to the cells arrested at S or M phase, autophagy was still induced, indicating that cell cycle arrest at G_1 is not prerequisite for induction of autophagy (Fig. 3).

In our laboratory, 14 autophagy defective mutants, called *apg* mutants, have been isolated (22). Most of the *APG* genes have been cloned and sequenced; however, their sites of action in the autophagic process are still to be determined (25–27). We have investigated the effect of rapamycin on *apg* mutants by observing the accumulation of autophagic bodies in the presence of rapamycin. It was found that none of the *apg* mutants accumulated autophagic bodies, implying that autophagy was not induced (*panels D–F* in Fig. 1 show the representative three *apg* mutants, but the other *apg* mutants were not shown). Therefore, the sites of action of all *APG* gene products are presumed to be downstream of the site of action of Tor.

Next we investigated the effect of cAMP, which is thought to play some role in cell cycle control by nutrient availability (28). It has been reported that cells harboring a triple mutation in cAMP pathway (*pde1*, *pde2*, and *cyr1*) are able to respond to exogenously added 1 mM cAMP, because phosphodiesterases (Pde1p, Pde2p), which are thought to degrade exogenously added cAMP, are defective (20). This strain grown in the presence of cAMP showed the same phenotypes as mutants with an elevated activity of the cAMP pathway, such as low glycogen accumulation (20). In *pho8Δ60* cells having these mutations as well as in wild-type cells, nitrogen starvation induced autophagy (Fig. 4). When cAMP was added to the medium, the induction of autophagy in these cells was inhibited (Fig. 4). Furthermore, this inhibition was not overcome by rapamycin (Fig. 4). These results indicated that 1 mM cAMP is inhibitory for the induction of autophagy.

DISCUSSION

In this study, we showed that Tor function is involved in the induction of autophagy in yeast. Inactivation of Tor by rapamycin treatment or by using a temperature-sensitive allele of *TOR2* promoted autophagy even under nutrient-rich conditions. Although the kinase domain of Tor was shown to be necessary for G_1 cell cycle progression (19), it remains to be determined whether Tor possesses actual kinase activity. Therefore, the mechanism through which Tor transmits signals is still unknown. However, from our results, it would be reasonable to speculate that under nutrient-rich conditions, Tor functions by blocking a factor needed for initiation of autophagy. This model would be consistent with what is known in mammalian cells, where the condition for phosphorylation of the ribosomal protein S6 is inhibitory for autophagy (8). S6 phosphorylation is sensitive to rapamycin, suggesting that a rapamycin-sensitive factor is involved upstream of S6 phosphorylation and autophagy (8, 29). We assume that this factor is RAFT/FRAP, the mammalian counterpart of Tor, which mediates S6 kinase activity (29–32).

Baedet *et al*. reported that inactivation of Tor leads to several G_0/stationary phase responses, which are also produced by nitrogen starvation (15). We showed that cell cycle arrest at S or M phase does not induce autophagy, implying that cell cycle arrest by itself is not the cause of induction of autophagy. Morover, even after the cell cycle is arrested at S or M phase, autophagy was induced by rapamycin treatment. This means that G_1 arrest itself is not the cause of autophagy, and autophagy and G_1 arrest are induced in a parallel manner under starvation conditions by the inactivation of Tor. Baedet *et al.* reported that the loss of Tor function also causes an inhibition of translation and proposed that the inhibition of protein synthesis causes G_1 arrest (15). We observed that the cells treated with cycloheximide in YPD did not induce autophagy (data not shown). Moreover, we have found that treatment with cycloheximide is inhibitory for induction of autophagy under starvation conditions (10). Therefore, additional experiments are needed to investigate the role of mRNA translation in autophagy.

There is a report that the two Tor proteins are involved in the initiation of sporulation (33). Sporulation is usually induced only in starvation medium, but inhibition of Tor function by rapamycin or by expressing a kinase-inactive mutant leads to initiation of sporulation even in nutrient-rich medium (33). Autophagy-defective mutants do not sporulate (22), suggesting

that autophagy is prerequisite for sporulation. So our finding that Tors are involved in control of autophagy is consistent with the report (33) on the involvement of Tor in initiation of sporulation. Tor2p has been shown to signal to the actin cytoskelton via the small GTPase Rho (18, 34). Because this function is specific to Tor2p and insensitive to rapamycin (19), we assume that it is not involved in autophagy.

The Ras-cAMP pathway is involved both in G_1 progression and initiation of sporulation, although the cAMP level does not change with nitrogen source availability (28, 35). Our finding indicates that increasing the concentration of cAMP inhibits autophagy even in the presence of rapamycin. The inhibitory effect of cAMP is observed also in mammalian cells (7). Baedet *et al.* reported that G_1 arrest by rapamycin is not affected by hyperactivation of the Ras-cAMP pathway using *bcy1* or *ras2* mutants, suggesting that the Ras-cAMP pathway is independent of the Tor pathway to G_1 arrest (15). Thus, unlike the case of G_1 arrest, cAMP may have to be kept below a certain critical level to initiate autophagy. Too high a concentration of cAMP may perturb some factors involved in the induction of autophagy.

APG and other autophagy-relative genes were reported to function not only in autophagy but also in the transport of aminopeptidase I (API)[2] to the vacuole (36, 37). Unlike most of the vacuolar enzymes, API is delivered from cytosol to the vacuole via nonsecretory and vesicle-mediated pathway (38, 39). Although both API transport and autophagy involve cytoplasm to vacuole delivery, API transport has different characteristics from autophagy. For example, API is delivered even under nutrient-rich condition, and its transport is highly selective. In contrast, autophagy is induced only under starvation, and autophagic delivery of the cytosol is nonselective (36). Therefore a factor that switches between autophagy and API transport might exist. Our analysis with *apg* mutants indicated that all *APG* gene products function downstream of Tors. Tor might be a molecule that switches Apg function directly or indirectly between autophagy and API transport.

Tor is the first molecule identified in the signal pathway in autophagy. We have found significant similarities in the control mechanism for autophagy of mammalian cells and yeast. Further analysis of autophagy in yeast would help our understanding of the process in mammalian cells.

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[2] The abbreviation used is: API, aminopeptidase I.

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