

Autophosphorylation Within the Atg1 Activation Loop Is Required for Both Kinase Activity and the Induction of Autophagy in *Saccharomyces cerevisiae*

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ABSTRACT

Autophagy is an evolutionarily conserved degradative pathway that has been implicated in a number of physiological events important for human health. This process was originally identified as a response to nutrient deprivation and is thought to serve in a recycling capacity during periods of nutritional stress. Autophagy activity appears to be highly regulated and multiple signaling pathways are known to target a complex of proteins that contains the Atg1 protein kinase. The data here extend these observations and identify a particular phosphorylation event on Atg1 as a potential control point within the autophagy pathway in *Saccharomyces cerevisiae*. This phosphorylation occurs at a threonine residue, T226, within the Atg1 activation loop that is conserved in all Atg1 orthologs. Replacing this threonine with a nonphosphorylatable residue resulted in a loss of Atg1 protein kinase activity and a failure to induce autophagy. This phosphorylation required the presence of a functional Atg1 kinase domain and two known regulators of Atg1 activity, Atg13 and Atg17. Interestingly, the levels of this modification were found to increase dramatically upon exposure to conditions that induce autophagy. In addition, T226 phosphorylation was associated with an autophosphorylated form of Atg1 that was found specifically in cells undergoing the autophagy process. In all, these data suggest that autophosphorylation within the Atg1 activation loop may represent a point of regulatory control for this degradative process.

MACROAUTOPHAGY (hereafter referred to as autophagy) is a highly conserved process of self-degradation that is essential for cell survival during periods of nutrient limitation (TSUKADA and OHSUMI 1993). During autophagy, a double membrane grows out from a specific nucleation site, known as the pre-autophagosomal structure, or PAS, in *Saccharomyces cerevisiae* and the phagophore assembly site in mammals (SUZUKI and OHSUMI 2007). This membrane encapsulates bulk protein and other constituents of the cytoplasm and ultimately targets this material to the vacuole/lysosome for degradation (XIE and KLIONSKY 2007). Recent studies have linked this pathway to a number of processes important for human health, including tumor suppression, innate immunity, and neurological disorders, like Huntington's disease (RUBINSZTEIN *et al.* 2007; LEVINE and KROEMER 2008). Determining how this pathway is regulated is therefore important for our understanding of these processes and our attempts to manipulate autophagy in clinically beneficial ways.

Supporting information available at <http://www.genetics.org/cgi/content/full/genetics.110.116566/DC1>.

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Most of the molecular components of the autophagy pathway were initially characterized in the budding yeast, *S. cerevisiae*, but orthologs of many of these Atg proteins have since been found in other eukaryotes (TSUKADA and OHSUMI 1993; MEIJER *et al.* 2007). A complex of proteins that contains the Atg1 protein kinase is of special interest and appears to be a key point of regulatory control within this pathway (KAMADA *et al.* 2000; BUDOVSKAYA *et al.* 2005; HE and KLIONSKY 2009; STEPHAN *et al.* 2009). In *S. cerevisiae*, genetic and biochemical data indicate that this complex is targeted by at least three different signaling pathways. Two of these pathways, involving the Tor and cAMP-dependent protein kinases, inhibit this process, whereas the AMP-activated protein kinase is needed for the full induction of autophagy (NODA and OHSUMI 1998; WANG *et al.* 2001; BUDOVSKAYA *et al.* 2004; STEPHAN and HERMAN 2006; KAMADA *et al.* 2010). The manner in which these signaling pathways regulate Atg1 activity and the precise role of this kinase in the autophagy process are presently matters of intense scrutiny.

Although Atg1 kinase activity is required for the induction of autophagy, relatively little is known about how this enzyme is regulated *in vivo*. Two proteins associated with Atg1, Atg13 and Atg17, have been shown to be required for full Atg1 kinase activity both *in vitro*

and *in vivo* (KAMADA *et al.* 2000; STEPHAN *et al.* 2009). The roles of these proteins appear to be conserved through evolution as functional homologs of both have been identified in fruit flies and/or mammals (HARA *et al.* 2008; CHAN *et al.* 2009; CHANG and NEUFELD 2009; GANLEY *et al.* 2009; HOSOKAWA *et al.* 2009; JUNG *et al.* 2009; MERCER *et al.* 2009). However, it is not yet clear precisely how these proteins stimulate Atg1 activity. In this study, we show that Atg1 is autophosphorylated within the activation loop and that this phosphorylation is required for both Atg1 kinase activity and the induction of autophagy. The activation loop is a structurally conserved element within the kinase domain and phosphorylation within this loop is often a necessary prerequisite for efficient substrate binding and/or phosphotransfer in the catalytic site (JOHNSON *et al.* 1996; NOLEN *et al.* 2004). This loop generally corresponds to the sequence between two signature elements within the core kinase domain, the DFG and APE motifs (HANKS and HUNTER 1995). Phosphorylation within this loop tends to result in a more ordered structure for this region and the proper positioning of key elements within the catalytic core of the kinase domain (KNIGHTON *et al.* 1991; JOHNSON and O'REILLY 1996; HUSE and KURIYAN 2002). We found that Atg1 activation loop phosphorylation was correlated with the onset of autophagy and that replacing the site of phosphorylation with a phosphomimetic residue led to constitutive Atg1 autophosphorylation *in vivo*. In all, the data here suggest that Atg1 phosphorylation within its activation loop may be an important point of regulation within the autophagy pathway and models that discuss these data are presented.

MATERIALS AND METHODS

Strains and growth media: Standard *Escherichia coli* growth conditions and media were used throughout this study. The yeast rich-growth medium, YPAD, consists of 1% yeast extract, 2% Bacto-peptone, 500 mg/liter adenine-HCl, and 2% glucose. The yeast YM glucose and SC glucose minimal growth media have been described (KAISER *et al.* 1994; CHANG *et al.* 2001). The nitrogen starvation medium, SD-N, consists of 0.17% yeast nitrogen base lacking amino acids and ammonium sulfate, and 2% glucose. Growth media reagents were from Difco (Detroit). Standard yeast genetic methods were used in this study. The yeast strains used were TN125 (*MATa ade2 his3 leu2 lys2 trp1 ura3 pho8::pho8Δ60*), YYK126 (TN125 *atg1Δ::LEU2*), YYK130 (TN125 *atg13Δ::TRP1*), PHY3687 (TN125 *atg1Δ::LEU2 atg13Δ::kanMX*), BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), PHY4167 (BY4741 *atg1Δ::kanMX*), PHY4177 (BY4741 *atg13Δ::kanMX*), and PHY4181 (BY4741 *atg17Δ::kanMX*) (NODA *et al.* 1995; KAMADA *et al.* 2000; GLAEVER *et al.* 2002).

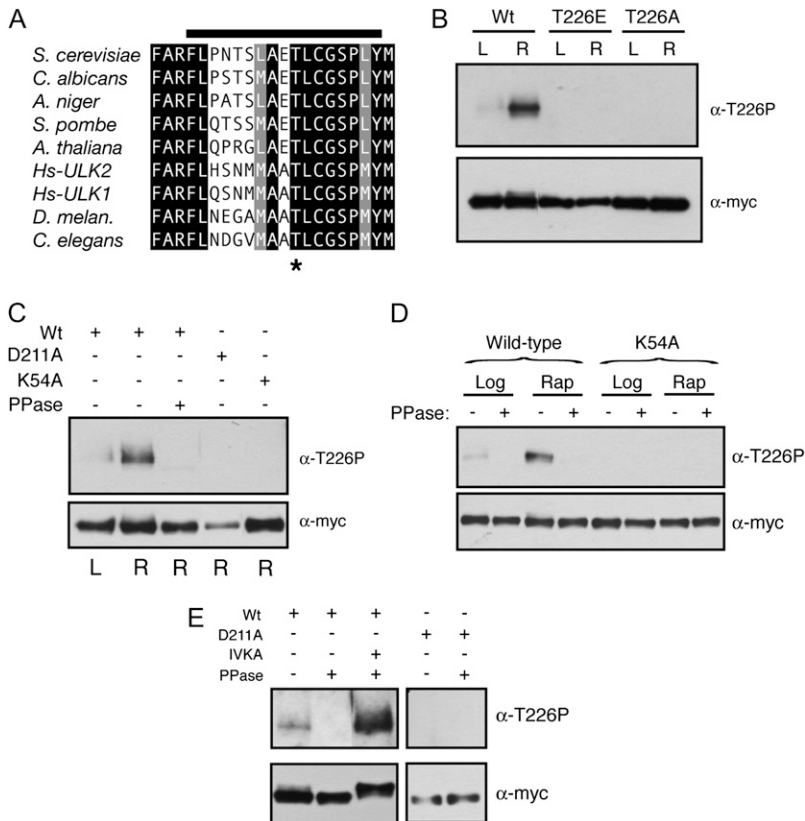
Plasmid constructions: Site-directed mutageneses were performed with the Clontech Transformer kit on the *ATG1*-containing plasmids, pPHY1115 and pPHY2376, to generate the variants (K54A, D211A, T226A, and T226E) used herein. Plasmid pPHY1115 was originally named pRS316-3xmycATG1 and was generously provided by Dr. Yoshinori Ohsumi.

Plasmid pPHY2376 is a pRS423-based construct in which Atg1 has three copies of the HA epitope at its N terminus; this plasmid was originally provided by Dr. Daniel Klionsky. Variants in the YFP-Atg1 fusion background were generated similarly with the wild-type plasmid, pPHY2297, as the starting vehicle (BUDOVSKAYA *et al.* 2005). For the rAtg1 variants, fragments encoding residues 1–420 were PCR amplified from the appropriate yeast plasmids and cloned into the pET21a expression vector. The plasmid, pPHY2427, encoded an HA epitope-tagged version of Atg13 under the control of the promoter from the copper-inducible *CUP1* gene. The *ATG13* locus was cloned from the plasmid, pRS316-ATG13, that was kindly provided by Dr. Takeshi Noda. A site-directed mutagenesis was performed to insert an *Xma*I site immediately following the start codon of *ATG13*. The *ATG13* coding sequences and transcriptional terminator were then cloned as a 3.1 kb *Xma*I-*Not*I fragment into pPHY2203 to form pPHY2427. The pPHY2203 plasmid was described previously and contains the *CUP1* promoter and the HA epitope (DEMINOFF *et al.* 2006).

Western blotting and immunoprecipitation: Protein samples for Western blotting were prepared as described (BUDOVSKAYA *et al.* 2005). The proteins were separated on SDS-polyacrylamide gels, transferred to nitrocellulose membranes and the membranes were probed with the appropriate primary and secondary antibodies. The Supersignal chemiluminescent substrate (Pierce Chemical, Rockford, IL) or Odyssey infrared imaging system (LI-COR Biosciences) was subsequently used to detect the reactive bands or fluorescence intensities. Immunoprecipitations were performed as described (BUDOVSKAYA *et al.* 2005; DEMINOFF *et al.* 2009). HA-tagged proteins were generally precipitated on anti-HA matrix beads (Roche) whereas myc-tagged proteins were immunoprecipitated with a monoclonal anti-myc antibody and then collected on Protein A-Sepharose (GE Healthcare) as described (BUDOVSKAYA *et al.* 2005; DEMINOFF *et al.* 2006). The amount of immunoprecipitated and co-immunoprecipitated proteins were subsequently assessed by Western immunoblotting with the appropriate antibodies. To assess phosphorylation of position T226 within Atg1, a polyclonal antiserum was generated in rabbits against a phosphorylated peptide, FLPNTSLAE(pT)LCGSPLY, that corresponds to the sequence of the Atg1 activation loop (see Figure 1A). The peptide synthesis, and antibody generation and purification was performed by Lampire Biological Laboratories. Western blots with this antibody were generally performed with cells overexpressing Atg1 from a 2 μ plasmid.

***In vitro* kinase assays (IVKAs):** The immunoprecipitated Atg1 proteins, or purified recombinant Atg1 fragments, were incubated for 30–60 min at 30° with 10 μ Ci [γ -³²P]ATP in a 40- μ l reaction (50 mM potassium phosphate, 5 mM NaF, 10 mM MgCl₂, 4.5 mM DTT, protease inhibitors, and phosphatase inhibitors) with or without 10 μ g of myelin basic protein (MBP) (Sigma, St. Louis). The reactions products were separated on SDS-polyacrylamide gels and the gels were fixed, dried, and analyzed either by autoradiography or by phosphorimaging with a Typhoon Trio (GE Healthcare).

Recombinant protein purification: *E. coli* cells were grown to an OD₆₀₀ of ~0.5/ml and IPTG was added to a final concentration of 1 mM to induce expression of the rAtg1 proteins. The induction was carried out for 4 hr. The cells were collected by centrifugation and lysed by sonication in lysis buffer (50 mM sodium phosphate, 500 mM NaCl, and protease inhibitors). Clarified cell lysates were then incubated with 1 ml Ni-NTA agarose beads (Qiagen) at 4° overnight. The beads were collected by centrifugation and washed three times with PBS containing 20 mM imidazole, and the recombinant proteins were eluted with PBS containing 250 mM imidazole.



Atg1. (E) The T226 phosphorylation signal was regenerated in an *in vitro* kinase assay. The indicated Atg1 proteins were precipitated from yeast cells that had been treated with rapamycin, and the relative level of T226 phosphorylation was assessed by Western blotting with the phosphospecific antibody. Where indicated, the precipitated Atg1 proteins were treated with λ phosphatase (PPase) and then subjected to an *in vitro* kinase autophosphorylation assay (IVKA).

Autophagy assays: Autophagy activity was assessed with several different assays that have been described previously. In general, autophagy was induced by treating mid-log-phase cells with 200 ng/ml rapamycin for the indicated times. The ALP-based assay measures the delivery and subsequent activation in the vacuole of an altered form of the Pho8 alkaline phosphatase, known as Pho8 Δ 60 (NODA *et al.* 1995; NODA and KLIONSKY 2008). The relative level of autophagy was indicated by the difference in alkaline phosphatase activity detected between extracts prepared from log-phase and rapamycin-treated cells. The data presented represent the average of at least three independent experiments where the autophagy levels generally varied by less than 15% between the different experimental replicates. In the GFP-Atg8 assay, the relative level of free GFP generated by proteolysis in the vacuole is used as an indicator of autophagy activity (SHINTANI and KLIONSKY 2004). The GFP-Atg8 fusion protein and the free GFP were detected by Western blotting with an anti-GFP antibody. The final assay assesses the processing of overexpressed aminopeptidase I, or Ape1, that occurs via the autophagy pathway (BUDOVSKAYA *et al.* 2004). In this assay, the cells express Ape1 at a level that saturated the cytoplasm-to-vacuole, or Cvt, transport system. Previous work has shown that the precursor Ape1 protein in these cells is processed by the autophagy pathway following either rapamycin treatment or nitrogen starvation (BUDOVSKAYA *et al.* 2004). The Ape1 proteins were detected by Western blotting with an anti-Ape1 antibody provided by Dr. Daniel Klionsky.

Fluorescence microscopy: The CFP-Atg11 and YFP-Atg1 fusions were under the control of the inducible promoter

from the yeast *CUP1* gene. Expression of the fusion proteins was induced by the addition of 100 μ M CuSO₄ for 2 hr at 30 $^{\circ}$, and the samples were imaged as described (BUDOVSKAYA *et al.* 2005).

RESULTS

Residue T226 within the activation loop was a site of Atg1 autophosphorylation: The activation loop of the *S. cerevisiae* Atg1 contains a candidate site of phosphorylation at threonine-226 (T226). This position, and much of the surrounding sequence, is conserved in Atg1 orthologs (Figure 1A). To test whether T226 was indeed a site of phosphorylation, we generated an antibody that specifically recognized the phosphorylated form of a peptide whose sequence corresponded to that of the Atg1 activation loop (Figure 1A; see MATERIALS AND METHODS). This antibody recognized the wild-type Atg1 protein but not the Atg1^{T226A} and Atg1^{T226E} variants that had substitutions at position T226 (Figure 1B). Interestingly, the signal with this antibody was found to increase upon exposure to conditions that lead to an induction of autophagy, such as rapamycin treatment (Figure 1B; see below). The recognition by this antibody was lost upon phosphatase treatment of the immunoprecipitated Atg1 and was absent from cells expressing

FIGURE 1.—T226 within the activation loop was a site of Atg1 autophosphorylation. (A) The activation loop sequences for the Atg1 orthologs from the indicated organisms are shown. Residues identical in all the orthologs are shown on a black background and those residues that are chemically similar are shown on a gray background. The T226 residue is indicated by an asterisk and the bar at the top indicates the peptide used to generate the phosphospecific antibody. Hs, *Homo sapiens*. (B) Phosphorylation at position T226 within the Atg1 activation loop increased upon rapamycin treatment. Note that the Atg1^{T226A} and Atg1^{T226E} variants were not recognized by the anti-T226P phosphospecific antibody. In B–E, the indicated Atg1 proteins were immunoprecipitated from cell extracts with an antibody directed against the indicated epitope, separated on SDS-polyacrylamide gels, and then analyzed by Western blotting with the indicated antibodies. The cell extracts were prepared from either log-phase (L) or rapamycin-treated (R) cultures. The protein signals were assessed by autoradiography following chemiluminescence illumination. (C and D) Atg1 recognition by the T226-P phosphospecific antibody was lost upon phosphatase treatment and was absent from cells that contained kinase-defective variants of Atg1. Where indicated, the precipitated Atg1 proteins were treated with λ phosphatase (PPase) prior to SDS-polyacrylamide gel electrophoresis. K54A and D211A refer to kinase-defective variants of

only the kinase-defective variants, Atg1^{K54A} or Atg1^{D211A} (Figure 1, C and D). The positions altered in these variants, K54 and D211, are highly conserved in protein kinases and are important for the proper positioning of ATP within the active site (ZOLLER *et al.* 1981; HANKS *et al.* 1988; ADAMS 2001). These results suggested that the recognition by this antibody required the presence of a phosphate group at position T226 and that the addition of this phosphate might be catalyzed by Atg1 itself. To examine this latter possibility further, we attempted to regenerate the phosphorylation signal at T226 *in vitro* with immunoprecipitated Atg1 proteins that had been pretreated with λ phosphatase. We found that the T226 phosphorylation was restored with the wild-type Atg1 but not with kinase-defective variants (Figure 1E; data not shown). Altogether, these data suggested that position T226 within the activation loop was a site of Atg1 autophosphorylation and that the presence of this modification was coincident with the induction of autophagy.

Phosphorylation at position T226 within the activation loop was required for Atg1 protein kinase activity:

To assess the importance of activation loop phosphorylation for Atg1 function, we replaced T226 with a nonphosphorylatable alanine residue and assessed Atg1 kinase activity both *in vivo* and *in vitro*. The *in vitro* assays examined Atg1 autophosphorylation and the phosphorylation of a nonphysiological substrate, the MBP (MATSUURA *et al.* 1997; KAMADA *et al.* 2000). In each case, the Atg1 proteins examined were precipitated from rapamycin-treated cells. We found that this Atg1^{T226A} variant exhibited significantly diminished activity, relative to the wild-type enzyme, in both of these assays (Figure 2, A–D). Two kinase-defective versions of Atg1, Atg1^{K54A} and Atg1^{D211A}, were used as negative controls in these experiments and the level of kinase activity associated with Atg1^{T226A} was similar to that observed with these control proteins. We also found that a pre-incubation of the precipitated Atg1 with ATP resulted in elevated kinase activity toward MBP (Figure 2C). This stimulation required the presence of the kinase domain and suggested that Atg1 autophosphorylation might increase the specific activity of this enzyme (supporting information, Figure S1, A and B). This autophosphorylation could occur at position T226 and/or other positions within the Atg1 protein.

To assess Atg1 kinase activity *in vivo*, we took advantage of previous observations indicating that Atg1 autophosphorylation results in the production of a slower-migrating form of this protein on SDS-polyacrylamide gels (Atg1-P in Figure 3A) (KAMADA *et al.* 2000; BUDOVSKAYA *et al.* 2005; STEPHAN *et al.* 2009). Our data indicate that the site of autophosphorylation responsible for this shift is distinct from T226 (see below and our unpublished observations). The relative level of this second, upper band can serve as a measure of the Atg1 kinase activity present in cells (STEPHAN *et al.* 2009). Consistent with

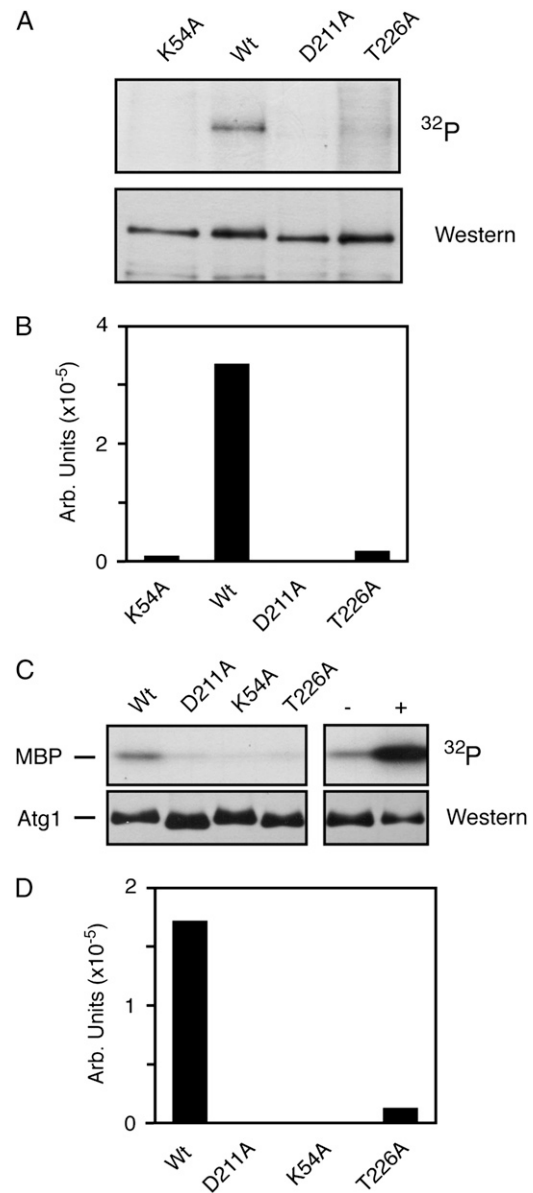


FIGURE 2.—The presence of T226 within the Atg1 activation loop was required for protein kinase activity. Wt, wild-type. (A) Replacing T226 with a nonphosphorylatable residue resulted in a loss of Atg1 autophosphorylation. Atg1 proteins with the indicated alterations were immunoprecipitated from rapamycin-treated cells and subjected to an *in vitro* autophosphorylation assay as described in the MATERIALS AND METHODS. The Atg1^{K54A} and Atg1^{D211A} variants were kinase-defective controls. In both A and B, the Atg1 proteins were tagged at the N terminus with three copies of the myc epitope. (B) Quantification of the phosphorimager data shown in A. (C) An *in vitro* kinase assay, using the MBP as substrate, was performed with the indicated Atg1 proteins isolated from rapamycin-treated cells. In the two right-hand lanes, the wild-type Atg1 protein was immunoprecipitated and either mock treated (–) or pre-incubated with 3 mM ATP for 60 min at 30°C (+). The samples were subsequently washed to remove the excess ATP and an *in vitro* kinase assay was performed with [γ -³²P]ATP and MBP. (D) Quantification of the phosphorimager analysis data in C.

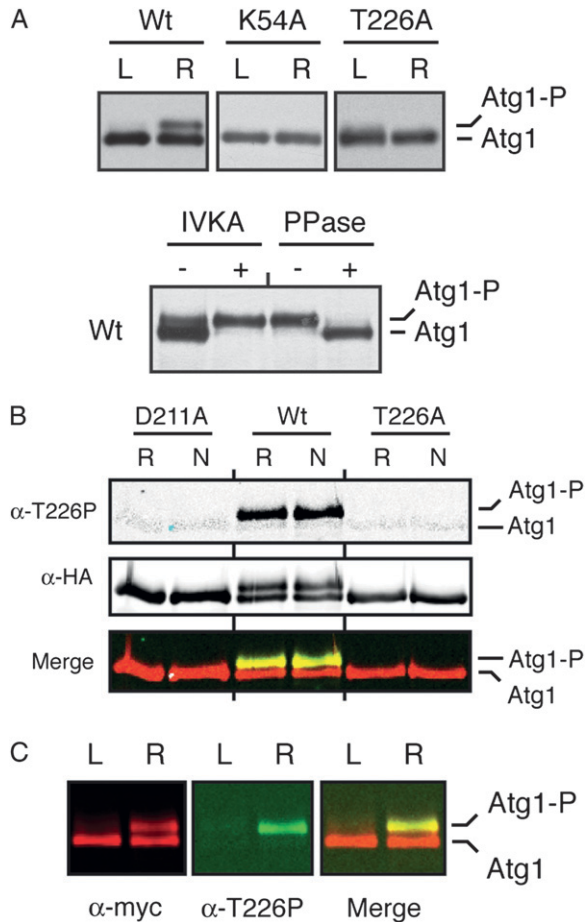


FIGURE 3.—The Atg1^{T226A} variant was defective for Atg1 autophosphorylation *in vivo*. (A) The presence of the T226A alteration resulted in a loss of Atg1 autophosphorylation *in vivo*. Western blots were performed with extracts prepared from either log-phase (L) or rapamycin-treated (R) cells with the indicated Atg1 proteins. In the bottom part, immunoprecipitated wild-type Atg1 was either mock treated (IVKA⁻) or subjected to an *in vitro* autophosphorylation reaction with 3 mM ATP (IVKA⁺). An aliquot of this latter sample was then split into two and either mock treated (PPase⁻) or incubated with λ phosphatase (PPase⁺). PPase, λ phosphatase; IVKA, *in vitro* kinase assay; Atg1-P, autophosphorylated form of Atg1 that exhibits an altered mobility on SDS-polyacrylamide gels. (B) Atg1 activation loop phosphorylation was elevated in response to nitrogen starvation and required the presence of a functional Atg1 kinase domain. In B and C, the indicated Atg1 proteins were immunoprecipitated from cell extracts with an antibody directed against the indicated epitope, separated on SDS-polyacrylamide gels, and then analyzed by Western blotting with the indicated antibodies. The cell extracts were prepared from either log-phase (L), rapamycin-treated (R) or nitrogen starved (N) cultures. The protein signals were detected with a LI-COR Odessey infrared detection system. For nitrogen starvation, log-phase cells were transferred to SD-N medium for 6 hr. D211A, a kinase-defective variant of Atg1. (C) The anti-T226P phosphospecific antibody (α -T226P) recognized a slower-migrating, autophosphorylated form of Atg1 that appeared upon rapamycin treatment. The protein signals were detected with a LI-COR Odessey infrared detection system and the right-most part shows a merged image for the α -myc and α -T226P signals.

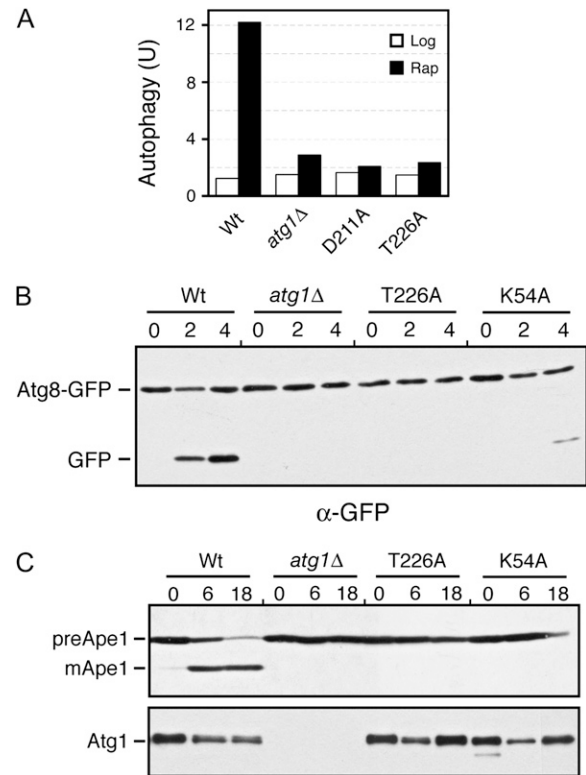


FIGURE 4.—The Atg1^{T226A} variant was defective for autophagy. (A) Autophagy activity was assessed with the ALP Pho8 Δ 60-based assay using an *atg1* Δ strain expressing the indicated Atg1 proteins. The *atg1* Δ control strain contained a vector plasmid. Extracts were prepared from cells that had been treated with 200 ng/ml rapamycin for either 0 (Log) or 4 hr (Rap). The data shown are the average of two independent experiments where the values obtained varied by less than 15% between replicates. (B) Autophagy activity was assessed with a GFP-Atg8 processing assay as described in MATERIALS AND METHODS. The level of free GFP was an indicator of the relative autophagy activity present. (C) Autophagy activity was assessed with an assay measuring the vacuolar processing of overexpressed Ape1 as described in MATERIALS AND METHODS. The presence of the mature, processed Ape1 protein (mApe1) was an indicator of autophagy activity. preApe1, precursor Ape1. The bottom part shows the relative levels of the different myc-tagged Atg1 proteins examined. For B and C, cells were treated with 200 ng/ml rapamycin for the hours indicated.

the *in vitro* data above, we found significantly less of the Atg1^{T226A} variant, relative to the wild type, in the slower-migrating form following either rapamycin treatment or nitrogen starvation, two conditions that are known to induce an autophagy response (Figure 3, A and B). The relative activity of the Atg1^{T226A} variant again resembled the kinase-defective controls, Atg1^{K54A} and Atg1^{D211A}. Interestingly, we found that the α -T226P antibody specifically recognized this slower-migrating form of Atg1 on Western blots (Figure 3, B and C). In all, these data suggested that T226 phosphorylation within the activation loop was required for Atg1 kinase activity and was associated with an autophosphorylated

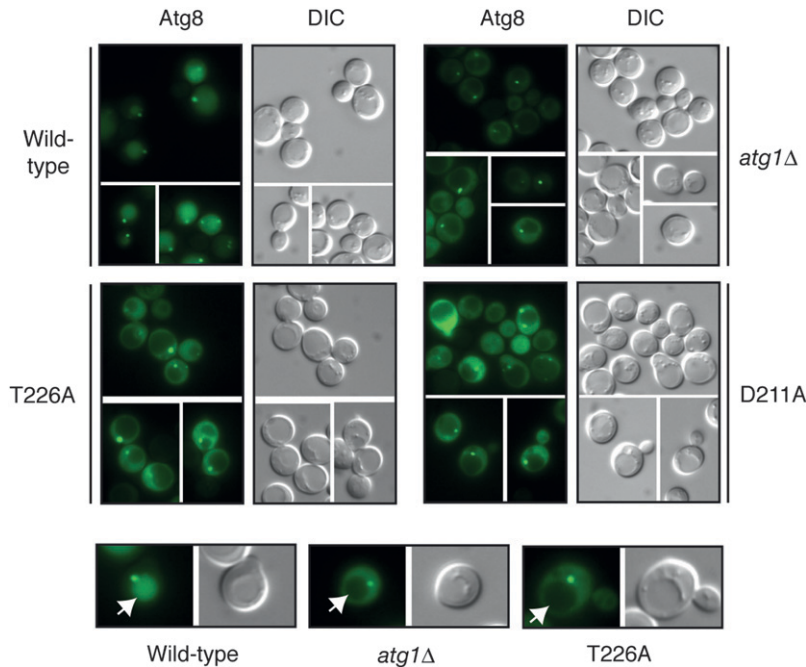


FIGURE 5.—An GFP-Atg8 fusion protein was not delivered to the vacuole in cells containing the Atg1^{T226A} variant. Yeast strains carrying the indicated Atg1 proteins were treated for 2 hr with 200 ng/ml rapamycin and the subcellular localization of an GFP-Atg8 fusion protein was then assessed by fluorescence microscopy. Atg8 is normally incorporated into the autophagosome and transported to the vacuolar compartment. This transport results in the presence of GFP fluorescence in the vacuoles of cells with a functional autophagy pathway. The vacuoles of the representative cells shown in the bottom row are indicated by the white arrows. Note that the GFP-Atg8 fusion was still found at the PAS in cells containing the Atg1^{T226A} variant.

form of Atg1 found specifically in cells undergoing autophagy.

Activation loop phosphorylation was required for the induction of autophagy: We used three different assays to test whether phosphorylation within the Atg1 activation loop was required for the induction of autophagy. The first assessed the vacuolar delivery and processing of an altered form of the Pho8 alkaline phosphatase, known as Pho8 Δ 60 (NODA *et al.* 1995). This truncated protein lacks a membrane-spanning domain and the targeting information necessary for normal delivery through the secretory pathway (KLIONSKY and EMR 1989; NODA *et al.* 1995). As a result, this protein can be delivered to the vacuole, and therein activated, only by the autophagy pathway. Using this assay, we found that no significant autophagy activity was detectable in cells carrying only the Atg1^{T226A} variant (Figure 4A). The levels of autophagy detected were similar to those associated with the *atg1* Δ control strain.

The second assay measured the processing of a GFP-Atg8 fusion protein (SHINTANI and KLIONSKY 2004). Atg8 is normally incorporated into the autophagosome and delivered into the vacuolar lumen (KIM *et al.* 2001; SUZUKI *et al.* 2001; SUZUKI and OHSUMI 2007). The Atg8 is then degraded by vacuolar hydrolases, thereby releasing the more stable GFP protein (SHINTANI and KLIONSKY 2004). The relative level of free GFP can be used as a measure of the autophagy activity present. As with the ALP-based assay, no significant processing of the GFP-Atg8 fusion was detected in cells with the Atg1^{T226A} variant (Figure 4B). This autophagy defect was corroborated by an examination of the subcellular localization of the GFP fluorescence in these cells. In wild-type cells, the GFP-Atg8 fusion is delivered to the vacuole and the free GFP accumulates within the lumen

of this organelle (Figure 5). In contrast, very little, if any, GFP fluorescence is detected in the vacuoles of mutants defective for autophagy (see *atg1* Δ in Figure 4) (SUZUKI *et al.* 2001). Consistent with the above processing defects, we found no significant GFP fluorescence within the vacuoles of *atg1* Δ cells carrying the Atg1^{T226A} variant (Figure 5). It should be noted that this GFP-Atg8 fusion was normally localized to the PAS in these cells (Figure 5).

Finally, we assessed the autophagy-dependent maturation of the Ape1 protein. Ape1 is a normal cargo for the cytoplasm-to-vacuole, or Cvt, transport pathway that is responsible for delivering a set of proteins to the vacuole during log-phase growth (KLIONSKY *et al.* 1992; KHALFAN and KLIONSKY 2002). Overexpression of Ape1 saturates the Cvt system and results in the cytoplasmic accumulation of an unprocessed, precursor form of this protein (see Figure 4C) (KLIONSKY *et al.* 1992). Upon the induction of autophagy, this protein is efficiently delivered to the vacuole and processed to its mature, active form (SCOTT *et al.* 1996; BUDOVSKAYA *et al.* 2004). We found that the Atg1^{T226A} variant was defective for this autophagy-mediated processing and that the Ape1 protein was present only in its precursor form in these cells (Figure 4C). Altogether, these data indicated that Atg1 autophosphorylation at T226 was necessary for the induction of the autophagy pathway in *S. cerevisiae*.

The Atg1^{T226E} variant exhibited constitutive autophosphorylation activity: To test whether the presence of T226 phosphorylation was sufficient for Atg1 autophosphorylation, we replaced this position with the potential phosphomimetic residues, glutamic and aspartic acid. Similar results were obtained with both substitutions, and we focus here on the Atg1^{T226E} variant. Interestingly, we found that this Atg1^{T226E} variant was

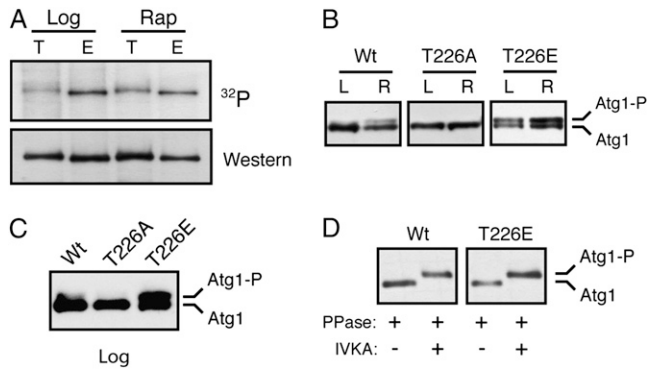


FIGURE 6.—The Atg1^{T226E} variant was a constitutively active protein kinase. (A) The Atg1^{T226E} variant exhibited elevated protein kinase activity in log-phase cell extracts. Atg1 was immunoprecipitated from either log-phase (Log) or rapamycin-treated (Rap) cells and subjected to an *in vitro* autophosphorylation assay with [γ - 32 P]ATP. T, wild-type Atg1; E, Atg1^{T226E} variant. The top is the autoradiograph image and the bottom is the Western blot control performed with an anti-myc antibody. For A–D, the Atg1 proteins were tagged with three copies of the myc epitope. (B) Atg1^{T226E} exhibited constitutive autophosphorylation *in vivo*. Western blots were performed with extracts prepared from either log-phase (L) or rapamycin-treated (R) cultures of an *atg1* Δ strain with the indicated Atg1 proteins. (C) The relative level of the slower-migrating form of the Atg1^{T226E} variant was elevated in log-phase cells. Western blotting was performed with log-phase extracts prepared from *atg1* Δ cells carrying the indicated Atg1 proteins. (D) The slower-migrating form of Atg1 was the result of an autophosphorylation at a position distinct from T226. The wild-type Atg1 and Atg1^{T226E} proteins were immunoprecipitated from rapamycin-treated cells and incubated with λ phosphatase. The sample was then split into two aliquots and either mock treated with the reaction buffer or incubated with 3 mM ATP. PPase, λ phosphatase; IVKA, *in vitro* kinase assay.

competent for autophosphorylation *in vitro* when isolated from rapamycin-treated cells (Figure S2, A). However, unlike the wild-type protein, Atg1^{T226E} also exhibited elevated levels of autophosphorylation in log-phase cells that were typically not undergoing autophagy. This constitutive, or unregulated, autophosphorylation activity was observed in both *in vitro* and *in vivo* assays (Figure 6, A–C). For example, in an *in vitro* assay, elevated levels of radioactivity, relative to the wild-type protein, were incorporated into the Atg1^{T226E} variant isolated from log-phase cells (Figure 6A). In addition, the fraction of this variant found in the slower-migrating form on SDS-polyacrylamide gels was elevated in log-phase cell extracts (Figure 6, B and C). In both assays, the overall level of autophosphorylation was slightly elevated with the Atg1^{T226E} variant. These *in vivo* results with the Atg1^{T226E} protein supported the above contention that the presence of the slower-migrating form of Atg1 was due to autophosphorylation at a site distinct from T226. In particular, we found that the upper band associated with the Atg1^{T226E} protein was lost upon phosphatase treatment and was regenerated in a subsequent incubation with ATP (Figure 6D and Figure S2, B).

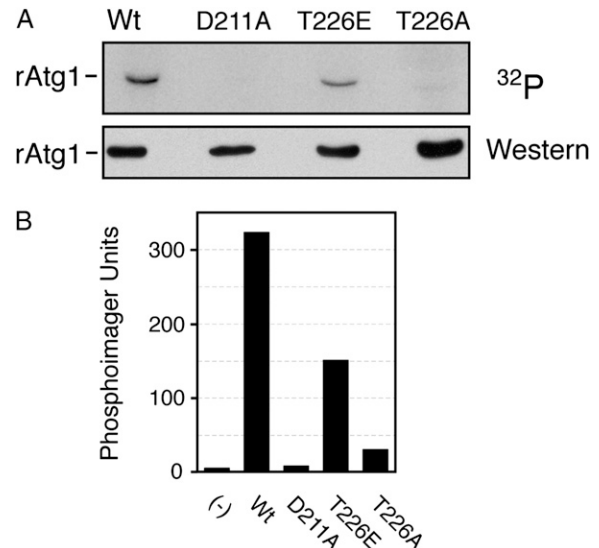


FIGURE 7.—Autophosphorylation of a recombinant Atg1 protein required the presence of position T226, the site of activation loop phosphorylation. (A) Recombinant fragments of Atg1 containing residues 1–420 were partially purified from *E. coli* cell extracts and incubated with [γ - 32 P]ATP to assess autophosphorylation *in vitro*. The relative level of label incorporation was then assessed with a phosphorimager and the image from this analysis is shown at the top. The bottom shows the Western blot control performed with an antibody specific to the His₆-tag present on these recombinant proteins. (B) Quantification of the phosphorimager analysis shown in A.

Finally, we found that a recombinant fragment of Atg1 produced in *E. coli* (rAtg1) was also capable of catalyzing an autophosphorylation reaction (Figure 7). This fragment contained residues 1–420 of the wild-type protein and included the entire protein kinase domain located at the N terminus of Atg1. This autophosphorylation was also dependent upon the presence of T226 as replacing this position with an alanine residue resulted in significantly diminished activity (Figure 7). This rAtg1^{T226A} variant also exhibited decreased activity in assays with MBP as substrate (Figure S3). In contrast, a recombinant protein with glutamic acid replacing T226 was capable of significant autophosphorylation (Figure 7). This result was similar to that obtained above with the wild-type protein and together these observations suggested that it was the phosphorylation at position T226 that was important for this Atg1 autophosphorylation. Moreover, the presence of a phosphomimetic at position 226 appeared to disrupt the normal control of Atg1 and resulted in constitutive autophosphorylation.

The Atg1^{T226E} variant was nonfunctional for autophagy: We also tested whether the Atg1^{T226E} and Atg1^{T226D} variants were functional for autophagy. Using the three processing assays described above, we found little, if any, autophagy activity in *atg1* Δ cells carrying these variants (Figure 8, A–C). Moreover, we found that the GFP–Atg8 fusion protein was not transported efficiently into the lumen of the vacuole in *atg1*–T226E cells (Figure 8D).

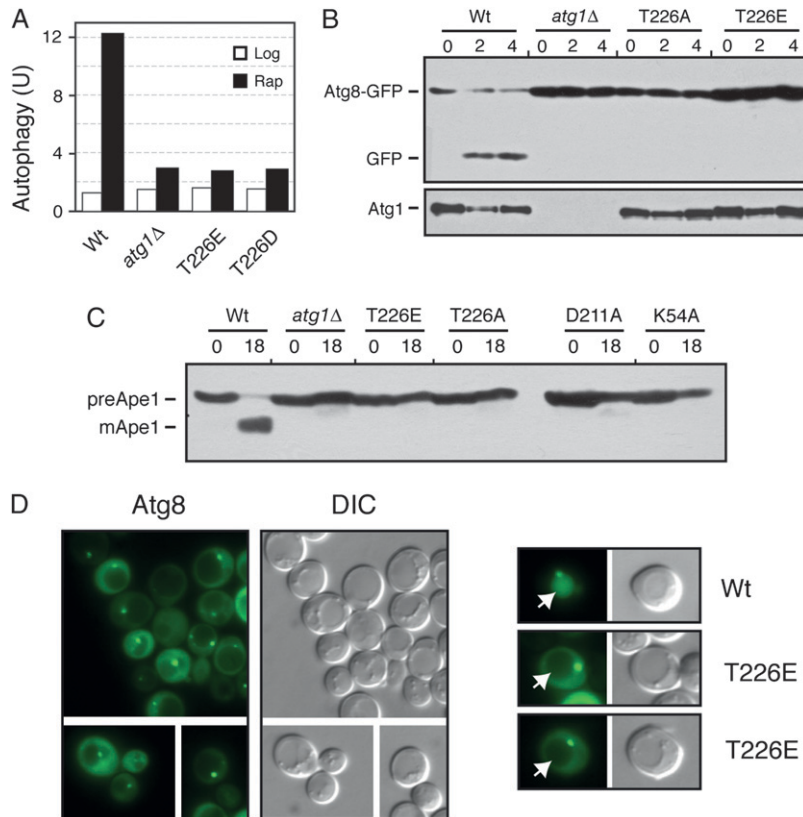


FIGURE 8.—The Atg1^{T226E} variant was defective for autophagy. (A) The ALP-based assay was used to assess autophagy activity in an *atg1Δ* strain expressing the indicated Atg1 proteins. Extracts were prepared from cells that had been treated with 200 ng/ml rapamycin for either 0 (Log) or 4 hr (Rap). The data shown are the average of two independent experiments where the values obtained varied by less than 15% between replicates. The *atg1Δ* control strain contained a vector plasmid. (B and C) Autophagy activity was assessed with either the GFP-Atg8 (B) or the Ape1 (C) processing assays as described in MATERIALS AND METHODS. In each case, the strains were treated with 200 ng/ml rapamycin for the indicated time (hours). B, bottom, shows the relative levels of the different Atg1 proteins examined. (D) A GFP-Atg8 fusion protein was not delivered to the vacuole in cells expressing only Atg1^{T226E}. The subcellular localization of an GFP-Atg8 fusion protein was assessed by fluorescence microscopy in *atg1Δ* cells carrying the indicated Atg1 proteins. The cells were treated with 200 ng/ml rapamycin for 2 hr prior to examination. Note that the GFP-Atg8 fusion was localized to the PAS in cells containing the Atg1^{T226E} variant.

This latter defect is consistent with a failure to carry out the normal autophagy process. Therefore, although these variants exhibited constitutive autophosphorylation activity, neither was capable of replacing the wild-type protein in the autophagy process. These variants also failed to induce autophagy in log-phase cells and had little detrimental effect upon autophagy when overexpressed in wild-type cells (Figure 8A and Figure S4). The autophagy defects did not appear to be due simply to gross misfolding as the Atg1^{T226E} protein was present at normal steady-state levels in yeast cells, was localized normally to the PAS, and interacted with both Atg13 and Atg17, two key regulators of Atg1 kinase activity (Figure 9, A–C) (KAMADA *et al.* 2000; KABEYA *et al.* 2005). However, we did find that the Atg1^{T226E} variant was less effective than the wild-type protein at phosphorylating the nonphysiological substrate, MBP, in an *in vitro* assay (Figure 9D). Therefore, it is possible that the autophagy defects observed with this variant might be due to a failure to phosphorylate an important, but as yet unknown, substrate *in vivo*.

Atg13 and Atg17 were required for T226 phosphorylation: The precise roles of Atg13 and Atg17 in the regulation of Atg1 kinase activity have not yet been defined. Here, we tested whether these regulators were required for the phosphorylation of Atg1 within its activation loop. Indeed, we found that T226 phosphorylation was greatly diminished in both *atg13Δ* and *atg17Δ* cells (Figure 10A). The residue levels remaining

in these mutants were higher than those observed in strains carrying only a kinase-defective version of Atg1 (Figure S5). This low level of T226 phosphorylation might be important for the suppression of *atg13Δ* defects observed upon the overexpression of Atg1 in these mutants (FUNAKOSHI *et al.* 1997). We also found that the overexpression of Atg13 resulted in an elevated level of T226 phosphorylation in both log-phase and rapamycin-treated cells (Figure 10B). Consistent with this observation, the overexpression of Atg13 also led to slightly increased levels of autophagy in rapamycin-treated cultures (Figure 10C). Finally, we tested whether the autophosphorylation associated with the Atg1^{T226E} variant required either Atg13 or Atg17. We found that the slower-migrating form of Atg1^{T226E} that was indicative of Atg1 kinase activity *in vivo* was still present in both *atg13Δ* and *atg17Δ* cells (Figure 10D). Therefore, the presence of a phosphomimetic within the Atg1 activation loop bypassed the normal requirement for these regulators for Atg1 autophosphorylation.

DISCUSSION

The autophagy pathway has been implicated in a wide variety of phenomena important for human health and has been suggested as a potential therapeutic target for a number of disease conditions (LEVINE 2005; RUBINSZTEIN *et al.* 2007; LEVINE and KROEMER 2008). To manipulate autophagy in a productive way, it is critical that we

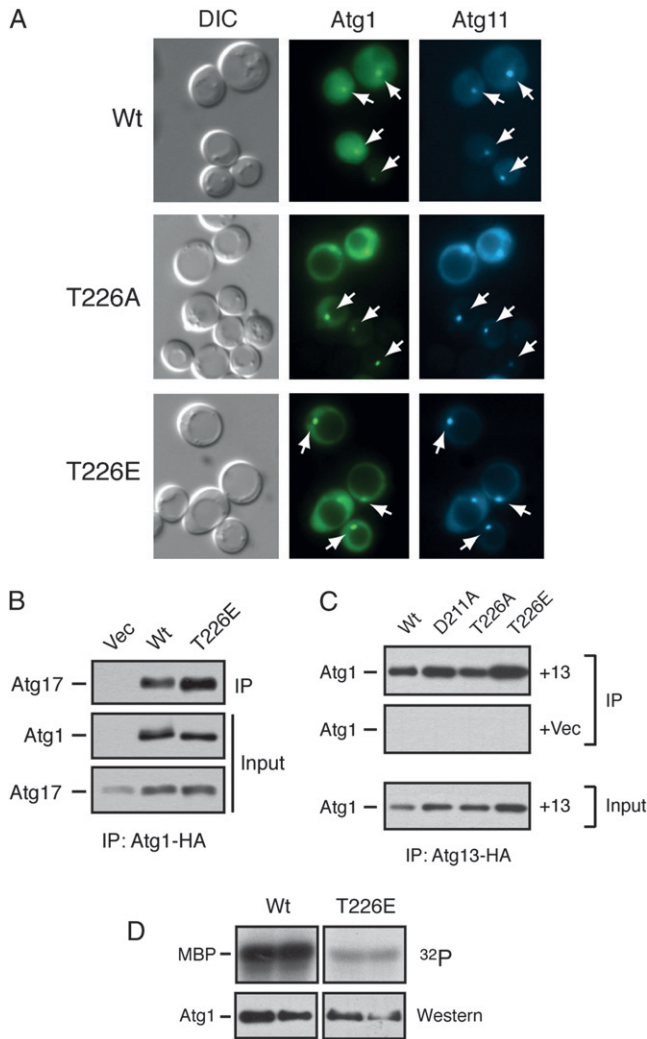


FIGURE 9.—The Atg1^{T226E} variant was localized to the PAS and interacted with Atg13 and Atg17. (A) The Atg1^{T226A} and Atg1^{T226E} proteins were localized normally to the PAS. The subcellular localization of YFP fusions to the indicated Atg1 proteins was assessed in an *atg1Δ* strain by fluorescence microscopy. An Atg11-CFP fusion protein was used as a marker for the PAS. The cells were all treated with 200 ng/ml rapamycin for 2 hr before examination. The white arrows indicate the location of the PAS in the respective cells. (B) The Atg1^{T226E} variant interacted with Atg17. The indicated HA-tagged Atg1 proteins were precipitated from cell extracts and the relative amount of associated Atg17 protein was assessed by Western blotting with an anti-myc antibody. (C) The Atg1^{T226E} variant interacted with Atg13. HA-tagged Atg13 was precipitated from cell extracts and the relative amounts of the associated Atg1 proteins were assessed by Western blotting with an anti-myc antibody. +13, cells containing the HA-tagged Atg13 protein; +Vec, cells containing a vector control and thus lacking the tagged Atg13 protein. (D) An *in vitro* kinase assay using MBP as substrate was performed with the wild-type Atg1 and the Atg1^{T226E} variant. The indicated Atg1 proteins were immunoprecipitated and incubated with [γ -³²P]ATP and the nonphysiological substrate, MBP; each sample was performed in duplicate. The bottom parts show the amount of Atg1 protein present in the *in vitro* reactions.

develop a thorough understanding of how this pathway is regulated *in vivo*. Toward this end, the work here identifies a specific phosphorylation event within the activation loop of Atg1 as a potential point of control for autophagy in *S. cerevisiae*. Several observations point to the importance of this phosphorylation at position T226 for the autophagy process. First, the substitution of a nonphosphorylatable alanine residue for the threonine normally present resulted in a loss of Atg1 kinase activity and a failure to induce the autophagy process. Second, a phosphospecific antibody, prepared against a phosphopeptide corresponding to the Atg1 activation loop, recognized the wild-type Atg1 but not the Atg1^{T226A} variant. Third, the relative level of T226 phosphorylation was found to increase specifically upon exposure to conditions that induce the autophagy process. Fourth, this antibody specifically recognized an autophosphorylated form of Atg1 that was present in cells undergoing autophagy. Fifth, we found that replacing the T226 position with an acidic residue, either glutamic or aspartic acid, resulted in an enzyme that exhibited constitutive activity with respect to autophosphorylation. This latter observation is important as it indicates that the presence of a potential phosphomimetic at position 226 in the activation loop results in the production of an active enzyme. Taken altogether, these data support the contention that Atg1 is phosphorylated within its activation loop and that this phosphorylation is important for the normal induction of the autophagy process. Since this site has been evolutionarily conserved in Atg1 orthologs, it is possible that this phosphorylation will be a point of control for autophagy in other eukaryotes.

Many protein kinases require phosphorylation within their activation loop for full activity and this modification can be a critical event in the activation of these enzymes (NOLEN *et al.* 2004). This phosphorylation can be catalyzed by a second enzyme or can be the consequence of an autophosphorylation reaction (LOCHHEAD *et al.* 2005; IMAJO *et al.* 2006; LOCHHEAD *et al.* 2006; PIRRUCCELLO *et al.* 2006; OLIVER *et al.* 2007). For Atg1, the data here suggest that this modification is self-catalyzed. A functional Atg1 kinase domain was required for T226 phosphorylation *in vivo* and this modification occurred *in vitro* with Atg1 immunoprecipitates. This phosphorylation was also shown to require the presence of Atg13 and Atg17, two previously identified regulators of Atg1 kinase activity. Interestingly, replacing position T226 with a phosphomimetic, such as glutamic acid, resulted in an enzyme that no longer required Atg13 or Atg17 for autophosphorylation *in vivo*. This bypass of the requirement for these regulators may suggest that facilitating Atg1 autophosphorylation within the activation loop is one of the functions of the Atg13 and Atg17 proteins during the autophagy process.

Although the data here suggest that T226 phosphorylation is required for the induction of autophagy,

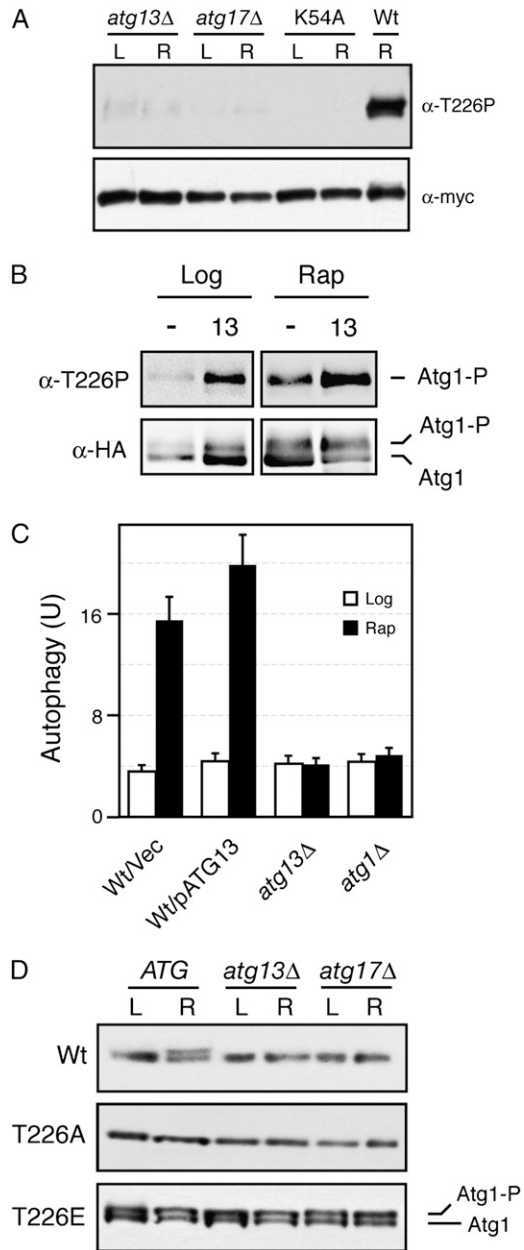


FIGURE 10.—Atg13 and Atg17 were required for Atg1 auto-phosphorylation within the activation loop. (A) T226 phosphorylation in *atg13Δ* and *atg17Δ* mutants. The wild-type Atg1 was precipitated from *atg13Δ*, *atg17Δ*, or wild-type cells and analyzed by Western blotting with the anti-T226P phosphospecific antibody. The Atg1^{K54A} variant (K54A) was used as a kinase-defective control. L, log phase; R, rapamycin-treated. (B) Overexpression of Atg13 resulted in an increased level of T226 phosphorylation. The relative level of T226 phosphorylation was assessed in both log-phase and rapamycin-treated wild-type cells carrying either a vector control (–) or an *ATG13* overexpression plasmid. The wild-type Atg1 was immunoprecipitated from these cells with an antibody specific for the HA epitope present on this protein. (C) Overexpression of Atg13 resulted in elevated levels of autophagy. A plasmid overexpressing Atg13 or a vector control were introduced into wild-type cells, and autophagy levels were assessed with the Pho8Δ60 ALP-based assay. The cells were treated with 200 ng/ml rapamycin for 0 (Log) or 4 hr (Rap) before analysis. (D) Atg13 and Atg17 were not required for the autophosphor-

neither the Atg1^{T226E} nor the Atg1^{T226D} variant was capable of supporting this degradative process *in vivo*. The underlying basis for this defect is not yet known, but there are a number of potential explanations that can be considered. On one extreme is the possibility that it is the threonine itself at position 226, and not its phosphorylation, that is important for Atg1 function. We do not favor this possibility given the strong correlation between T226 phosphorylation and both the induction of autophagy and the presence of Atg1 kinase activity, as measured by autophosphorylation. In addition, this model is difficult to reconcile with the observation that the presence of a phosphomimetic at position 226 led to constitutive Atg1 autophosphorylation. Therefore, we feel that the results obtained here with the Atg1^{T226E} and Atg1^{T226D} variants require a different explanation. One possibility is that the phosphomimetic does not fully restore the functionality of a phosphorylated threonine and therefore results in an enzyme with only partial activity. This could explain why the Atg1^{T226E} protein is competent for autophosphorylation but not the phosphorylation of an exogenous substrate, like MBP. Alternatively, this failure to phosphorylate MBP might reflect some inherent aspect of Atg1 biology. For example, Atg1 might normally participate in a negative feedback loop that is permanently engaged in the Atg1^{T226E} variant. Another possibility is that the autophagy defects might be due to the inability of these variants to exist in a kinase-inactive form. Previous work has indicated that Atg1 has both kinase-dependent and kinase-independent activities in the autophagy process (ABELIOVICH *et al.* 2003; CHEONG *et al.* 2008; KAWAMATA *et al.* 2008). Atg1 may therefore need to oscillate between kinase-active and kinase-inactive states to stimulate autophagy. Clearly, further work will be necessary to distinguish between these and other potential explanations. However, we feel that the work here in its totality identifies the autophosphorylation of Atg1 within its activation loop as an important event in the normal induction of autophagy. Since Atg1 and its regulators have been conserved through evolution, a continued analysis of this modification is likely to provide additional insights into the general control of this degradative process.

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ylation of Atg1^{T226E} *in vivo*. Cell extracts were prepared from either log-phase (L) or rapamycin-treated (R) cultures of wild-type, *atg13Δ*, and *atg17Δ* cells carrying the indicated Atg1 proteins. The relative levels of the slower-migrating, autophosphorylated form of Atg1 were assessed by Western blotting with an anti-myc antibody. The Atg1 proteins assayed were all tagged with three copies of the myc epitope.

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