Plant Physiol. (1996) 111: 1233–1241

Autophagy in Tobacco Suspension-Cultured Cells in Response to Sucrose Starvation[1]

Yuji Moriyasu* and Yoshinori Ohsumi

Department of Biology, Faculty of International Relations, University of Shizuoka, 52–1 Yada, Shizuoka-shi, Shizuoka 422, Japan (Y.M.); and Department of Biology, College of Arts and Sciences, University of Tokyo, 3–8–1 Komaba, Meguro-ku, Tokyo 153, Japan (Y.O.)

The response of tobacco (*Nicotiana tabacum*) suspension-cultured cells (BY-2) to nutrient starvation was investigated. When the cells that were grown in Murashige-Skoog medium containing 3% (w/v) sucrose were transferred to the same medium without sucrose, 30 to 45% of the intracellular proteins were degraded in 2 d. An analysis with sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that proteins were degraded nonselectively. With the same treatment, protease activity in the cell, which was measured at pH 5.0 using fluorescein thiocarbamoyl-casein as a substrate, increased 3- to 7-fold after 1 d. When the cysteine protease inhibitor (2*S*,3*S*)-*trans*-epoxysuccinyl-L-leucylamido-3-methyl-butane (10 μM) was present in the starvation medium, both the protein degradation and the increase in the protease activity were effectively inhibited. Light microscopy analysis showed that many small spherical bodies accumulated in the perinuclear region of the cytosol 8 h after the start of the inhibitor treatment. These bodies were shown to be membrane-bound vesicles of 1 to 6 μm in diameter that contained several particles. Quinacrine stained these vesicles and the central vacuole; thus, both organelles are acidic compartments. Cytochemical enzyme analysis using 1-naphthylphosphate and β-glycerophosphate as substrates showed that these vesicles contained an acid phosphatase(s). We suggest that these vesicles contribute to cellular protein degradation stimulated under sucrose starvation conditions.

Almost all cellular proteins turn over (Huffaker and Peterson, 1974; Goldberg and St. John, 1976; Davies, 1982; Hershko and Ciechanover, 1982). In mammalian cells the mechanisms underlying cellular protein degradation are divided into lysosomal and nonlysosomal pathways. The lysosomal pathway is thought to be responsible for the degradation of long-lived cytosolic proteins and for enhanced protein degradation under stressed conditions. The contribution of the lysosomal pathway to the total cellular protein degradation has been shown to vary from as low as 10 to 30% to as high as 85 to 100% by different researchers using different cells under different conditions (Dice, 1987). In plant cells knowledge about the mechanism of protein degradation is extremely limited compared with that in animal cells. Recently, a ubiquitin-proteasome system, which is thought to be a main contributor of the nonlysosomal pathway in mammalian and yeast cells, has been shown to be present in plant cells (Vierstra, 1993). However, the existence of a lysosomal pathway in plant cells has not been demonstrated.

Autophagy may contribute to growth and differentiation of plants in many aspects. During plant development many phenomena accompanying autolysis occur at the cellular, tissue, and organ levels, including the autolysis of three cells of the four embryo sac cells during the formation of an embryo, the differentiation of tracheary elements during the formation of xylem, the autolysis of petal cells after fertilization, and the senescence of leaves (Noodén, 1988). Such autolysis may originate from cellular autophagy. Moreover, cellular autophagy was shown to occur during the formation of the vacuoles in root meristematic cells of *Euphorbia* (Marty, 1978). Although the elucidation of the mechanism of autophagy is an important issue in plant cell biology, research on this phenomenon is incomplete. Journet et al. (1986) investigated the metabolism in cultured plant (*Acer pseudoplatanus*) cells upon Suc starvation. They showed that net protein degradation does occur with Suc starvation and proposed that this protein degradation is due to autophagy. But the mechanism of this protein degradation was not examined.

The aim of this study was to elucidate the mechanism of autophagy in plant cells. We examined intracellular protein degradation in cultured tobacco (*Nicotiana tabacum*) cells under Suc starvation conditions. In mammalian and yeast cells autophagy has been known to be stimulated under nutrient starvation conditions (Schworer and Mortimore, 1979; Takeshige et al., 1992; Baba et al., 1994). In this paper we report that, when the cells cultured in MS medium containing Suc are transferred to MS medium without Suc, net degradation of cellular proteins occurs. We suggest that this degradation is accomplished through an autophagic process via the formation of autolysosomes.

MATERIALS AND METHODS

Tobacco (*Nicotiana tabacum*) suspension-cultured cells (BY-2) used in this study were maintained by passaging 1.3 mL of the cell suspension in stationary phase to 80 mL of fresh MS medium containing 3% (w/v) Suc every week.

[1] This work was supported by grants-in-aid for scientific research (no. 06740619, no. 07640867) from the Ministry of Education, Science and Culture of Japan to Y.M.

* Corresponding author; e-mail moriyasu@momo1.u-shizuoka-ken.ac.jp; fax 81–54–264–5099.

Abbreviations: E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; E-64c, (2*S*,3*S*)-trans-epoxysuccinyl-L-leucylamido-3-methyl-butane; FTC-casein, fluorescein thiocarbamoyl-casein; MeOH, methanol; MS medium, Murashige-Skoog medium.

Cultures were grown in flasks at 25 ± 1°C with a rotation of 100 rpm.

Suc Starvation

Four-day-old, logarithmically growing cells were collected by centrifugation at 100*g* for 4 min. Cell pellets were resuspended in MS medium without Suc, and after an additional centrifugation step the cells were suspended in the original volume of MS medium without Suc. The resultant cell suspension in the starvation medium was transferred to a Petri dish (35 or 60 mm in diameter) and kept at 25 ± 1°C with a rotation of 100 rpm. Protease inhibitors were added from stock solutions (×100) in MeOH, DMSO, or water.

Measurement of Cellular Fresh Weights and Cellular Protein

Cells in 1 mL of suspension were collected on a glass filter (GF/A, 24 mm in diameter, Whatman) and washed with 10 mL of water using vacuum filtration. The weight of the glass filter with cells was measured, and the fresh weight of the cells was calculated by subtracting the weight of a glass filter through which only culture medium had been passed. The filter containing the cells was transferred to a Petri dish (60 mm in diameter), in which the cells were released from the filter by 3 mL of 0.2 M NaOH. The cell suspension in 0.2 M NaOH was transferred to a plastic tube (16.2 × 103 mm) and homogenized with a homogenizer (Polytron model K with a generator shaft of 12 mm in diameter; Kinematica, Littau, Switzerland) for 15 s at maximal speed and subsequently with a sonicator (B-220J-1; Branson, Danbury, CT) for 30 s. The homogenate was centrifuged at 2000*g* for 20 min. The amount of protein in the supernatant was measured according to the method of Lowry et al. (1951), modified by Bensadoun and Weinstein (1976). BSA was used as a standard.

SDS-PAGE

Cells that were collected from 1 mL of suspension were washed with 10 mL of water on a glass filter, transferred to a Teflon homogenizer, and homogenized with 1 mL of 100 mM Hepes-Na (pH 7.5), 1 mM EDTA, 100 μM leupeptin, 1 mM PMSF, and 28 mM 2-mercaptoethanol. The homogenate was centrifuged at 15,000*g* for 10 min. The supernatant was mixed with the same volume of 2× SDS-PAGE sample preparation buffer (Tris-SDS-β-mercaptoethanol SepraSol; Daiichi Pure Chemicals, Tokyo, Japan) and boiled at 100°C for 90 s. Proteins were separated by electrophoresis on a 10 to 20% SDS-polyacrylamide gradient gel (Multi gel 10/20, Daiichi) with the discontinuous buffer system of Laemmli (1970) and visualized by silver staining. Phosphorylase b from rabbit muscle (97 kD), BSA (66 kD), aldolase from rabbit muscle (42 kD), carbonic anhydrase from bovine erythrocytes (30 kD), trypsin inhibitor from soybean (20 kD), and lysozyme from egg white (14 kD) were used as molecular mass marker proteins.

Protease Assay

Cells were collected from 3 mL of suspension by vacuum filtration through a glass filter (GF/A, 41 mm in diameter, Whatman) and washed with 30 mL of water. Cells were transferred from a filter to a mortar using a microspatula and homogenized in 0.5 mL of 0.1 M acetic-Na (pH 5.0) containing 28 mM 2-mercaptoethanol and 0.1 g of sea sands. The homogenization and the following procedure were done at 0 to 4°C. The homogenate was centrifuged at 15,000*g* for 10 min, and 60 μL of the resulting supernatant and 40 μL of 0.5% (w/v) FTC-casein in 5 mM Tris-Cl (pH 7.5) were incubated at 37°C for 30 min. In the experiments in which the effects of several protease inhibitors were examined, 60 μL of the preincubation mixture containing 30 μL of the supernatant, protease inhibitor, and water were kept at room temperature for 5 min before the enzyme reaction. The concentration of each inhibitor given in the text is the same as that in the preincubation mixture. After the preincubation the reaction was started by the addition of 40 μL of 0.5% (w/v) FTC-casein and continued at 37°C for 60 min. The reaction was stopped by the addition of 100 μL of 10% (w/v) TCA. After the reaction tubes were kept on ice for about 1 h, they were centrifuged at 15,000*g* for 5 min. The supernatant (150 μL) was centrifuged again at the same speed for 10 min. The supernatant from the second centrifugation (100 μL) was mixed with 2 mL of 0.5 M Tris-Cl (pH 8.5). Fluorescence at 525 nm was measured with an excitation wavelength at 490 nm using an F-1200 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The kinetics for the fluorescence production were almost linear under these assay conditions.

Light and Fluorescence Microscopy

Cells were observed on a Nikon Optiphoto microscope with a ×40 (NA = 0.70) objective lens and ×10 oculars, equipped with fluorescence and Nomarski differential interference contrast optics. Photographs were taken through a ×40 or ×20 (NA = 0.50) objective lens and a ×5 projection lens using a Nikon UFX purpose-built photomicrographic camera with Kodak TMAX ISO 400 film.

Vital Staining with Quinacrine or Neutral Red

Cells in 100 μL of suspension medium were washed by centrifugation through 5 mM Hepes-Na (pH 7.5) containing 0.1 M sorbitol. The resuspended cells were stained in the same solution containing 40 μM quinacrine or 35 μM neutral red for 5 min at room temperature. The cells were washed again with the same solution and observed.

Assay of Acid Phosphatase

A crude enzyme solution was prepared as described for the protease assay. The reaction mixture contained 250 μL of 0.1 M acetic-Na (pH 5.0), 50 μL of the cell homogenate, 190 μL of water, and 10 μL of 50 mM *p*-nitrophenylphosphate. The reaction was started by the addition of the substrate and continued at 37°C for 15 min. The reaction

was stopped by the addition of 0.8 mL of 1 M sodium carbonate, and the A_{405} was measured.

Enzyme Cytochemistry of Acid Phosphatase using a Light Microscope

Cells were fixed overnight with 1% (w/v) glutaraldehyde and 1% (w/v) formaldehyde in 0.1 M phosphoric-Na (pH 7.4) and 86 mM NaCl at 4°C. After the cells were washed with 0.1 M acetic-Na (pH 5.0), they were kept in 50 mM acetic-Na (pH 5.0) containing 0.1% (w/v) Fast Garnet GBC and 1 mM 1-naphthylphosphate for 5 to 10 min at room temperature and observed with a light microscope.

Enzyme Cytochemistry of Acid Phosphatase using an Electron Microscope

Cells were fixed overnight with 1% (w/v) glutaraldehyde and 1% (w/v) formaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C. The pH of the buffer was adjusted with HCl. After the cells were washed with 8% (w/v) Suc in 0.1 M sodium cacodylate buffer (pH 7.2) and subsequently with 8% (w/v) Suc in 50 mM acetic-Na (pH 5.0), they were kept in 45.5 mM acetic-Na (pH 5.0), 3 mM lead nitrate, 10 mM β-glycerophosphate, and 230 mM Suc for 20 min at room temperature. After the reaction the cells were washed with 8% (w/v) Suc in 0.1 M sodium cacodylate buffer (pH 7.2) and postfixed with 1% (w/v) osmium tetroxide at room temperature for 2 h. Cells were dehydrated in an ethanol series, followed by propylene oxide, and embedded in Spurr's resin. Thin sections of resin-embedded cells were prepared and stained with lead citrate. They were viewed in an electron microscope (LEM 2000; Akashi, Tokyo, Japan).

Reagents

Pepstatin A, leupeptin, antipain, and E-64 were purchased from Peptide Institute (Minoh-shi, Osaka, Japan). Sodium 1-naphthylphosphate, sodium β-glycerophosphate, and neutral red were from Wako Pure Chemical Industries (Osaka, Japan). Glutaraldehyde, osmium tetroxide, propylene oxide, sodium cacodylate, lead nitrate, and a Spurr's resin kit were from TAAB Laboratories Equipment (Reading, UK). Paraformaldehyde was from Koso Chemical (Tokyo, Japan). Fast Garnet GBC, *p*-nitrophenylphosphate di(Tris)salt, fluorescein isothiocyanate (isomer I), quinacrine, carbonyl cyanide *m*-chlorophenylhydrazone, and PMSF were from Sigma. Casein (according to Hammarsten) was from Merck (Darmstadt, Germany). BSA (fraction V-Cohn) was from Armour (Kankakee, IL). E-64c was a generous gift from M. Tamai (Taisho Pharmaceutical, Oomiya, Japan). All other reagents used in this work were of analytical grade. Stock solutions of E-64c were prepared in MeOH, and those of PMSF and pepstatin A were in DMSO. FTC-casein was prepared according to the method of Twining (1984). Formaldehyde used to fix the cells for enzyme cytochemical analysis was prepared from paraformaldehyde just before use.

RESULTS

Suc Starvation Induces Protein Degradation

When the 4-d-old cultured tobacco cells were transferred to fresh MS medium containing Suc, total cellular protein increased about 3-fold after 1 d (Fig. 1). In contrast, when the cells were transferred to MS medium without Suc, total cellular protein decreased (Fig. 1). These results indicate that Suc starvation induced bulk degradation of intracellular proteins. The rate of protein degradation accelerated 1 d after the start of Suc starvation, which is consistent with the result of Journet et al. (1986), who used cultured sycamore (*Acer pseudoplatanus*) cells. They reported that net protein degradation began 30 h after the start of Suc starvation, a time when intracellular carbohydrates (Suc plus starch) disappear. In cultured tobacco cells the net protein that degraded after 2 d of starvation amounted to 30 to 45% of the initial total protein. In contrast to the remarkable decrease in the total protein, the fresh weight of the cells decreased only slightly during starvation (Fig. 1).

Suc Starvation Induces Intracellular Protease Activity

Cellular protease activity per milliliter of culture increased 3- to 7-fold following 1 d of Suc starvation (Fig. 2A). Hence, protein degradation induced by Suc starvation was coordinated with an increase in protease activity. The specific activity of protease increased 3.3- to 7.8-fold, since the total cellular protein per milliliter of culture decreased to about 90% of the initial value after 1 d of Suc starvation (Fig. 1). In contrast, when the cells were transferred to MS medium with Suc, the specific activity of protease decreased to about 65% of the initial value after 1 d.

To characterize the increased protease(s), we examined the effects of several protease inhibitors. The Cys protease inhibitor E-64c (10 μM) inhibited about 70% of the total activity in both homogenates prepared from the cells be-

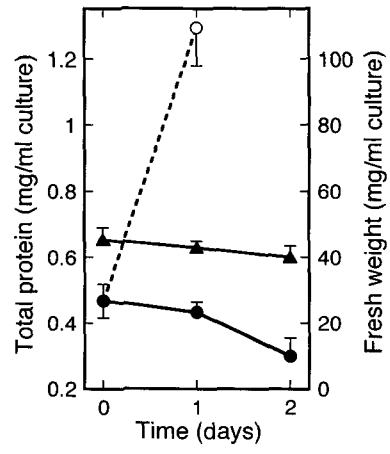

Figure 1. Intracellular protein degradation during Suc starvation in cultured tobacco cells. Total protein (●) and fresh weight (▲) of the cells in 1 mL of culture medium were measured for 2 d of Suc starvation. The change in total protein (○) of the cells in 1 mL of culture medium, when the cells were transferred to MS medium containing Suc, was also measured. Results are means ± SD (n = 5).

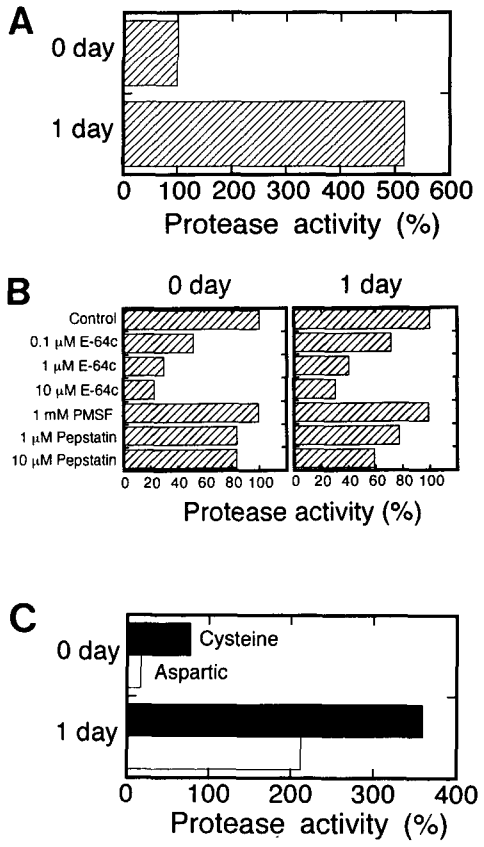

Figure 2. Induction of intracellular proteases by Suc starvation in cultured tobacco cells. Homogenates were prepared from the cells before (0 day) and 1 d after Suc starvation (1 day). A, Protease activities in these two homogenates (0 day and 1 day) were measured and are shown as relative activities per milliliter of culture medium. B, Effects of various protease inhibitors on FTC-casein-degrading activities in these two homogenates (0 day and 1 day). Values in A and B are the means of duplicate treatments in one experiment. Separate experiments were done three times, and similar results were obtained. C, The activation of a Cys (black bars) and an Asp (white bars) protease after 1 d of Suc starvation was redrawn from A and B, considering that the control value of 1 d in B was 5.2 times higher than that of 0 d in B. It was assumed that the activities inhibited by 10 μM E-64c and 10 μM pepstatin A were the activities of a Cys and an Asp protease, respectively.

fore and 1 d after starvation (Fig. 2B). The Asp protease inhibitor pepstatin A (10 μM) also inhibited a significant portion of the total activity in both homogenates (Fig. 2B). The Ser protease inhibitor PMSF did not have any effect in either homogenate (Fig. 2B). The activities of both Cys and Asp proteases increased 5- to 12-fold, respectively, after 1 d of Suc starvation, assuming that E-64c and pepstatin A are specific inhibitors of Cys and Asp proteases, respectively (Fig. 2C).

Cys Protease Inhibitors Block Both Protein Degradation and the Induction of Protease Activity

Since the above results suggested the involvement of Cys and Asp proteases in the enhanced protein degradation during starvation, we examined the effects of inhibitors to these proteases on various cell activities. When added to the starvation medium, E-64c effectively inhibited protein degradation, as shown in Figure 3. Another Cys protease inhibitor, leupeptin, also showed a similar effect (data not shown). In contrast to these two Cys protease inhibitors, pepstatin A did not have any apparent effects on protein degradation (data not shown). These three inhibitors did not affect cell viability, which was determined by observing cytoplasmic streaming and vital staining with fluorescein diacetate, during the 3-d treatment. In addition, we ascertained that E-64c did not affect the fresh weight during starvation (data not shown).

Next we analyzed cellular proteins by SDS-PAGE before and after starvation (Fig. 4). The band pattern did not change significantly, but the intensity of each band decreased during starvation (Fig. 4, MeOH versus t = 0). This result indicates that the decrease in the total protein comes not from the degradation of specific proteins but from the nonselective degradation of intracellular proteins. Treatment with E-64c and leupeptin rescued almost all of the polypeptides from degradation (Fig. 4, E-64c and Leupeptin).

We further examined whether E-64c, given from outside the cells, inhibited the induction of protease activity. After the cells were starved for 1 d in the presence of 10 μM E-64c, protease activity in the cell homogenate was measured. Treatment with E-64c completely abolished the increase in protease activity induced by Suc starvation (Fig. 5, E-64c versus MeOH). These results suggest that a Cys protease(s) plays an essential role in intracellular protein degradation induced by Suc starvation.

By contrast, treatment with 10 μM pepstatin A apparently did not affect the protease activity (Fig. 5, Pepstatin A versus DMSO). In the case of pepstatin A, however, it is possible that its inhibition cannot be measured with this method because pepstatin A is a reversible inhibitor. Another possibility is that pepstatin A does not penetrate the cells. In mammalian cells pepstatin A has been shown to be taken up very slowly by endocytosis (Seglen, 1983; Gordon and Seglen, 1989). We could not determine whether an Asp protease(s) contributes to the protein degradation induced by Suc starvation because of the absence of effective inhibitors for Asp proteases in situ.

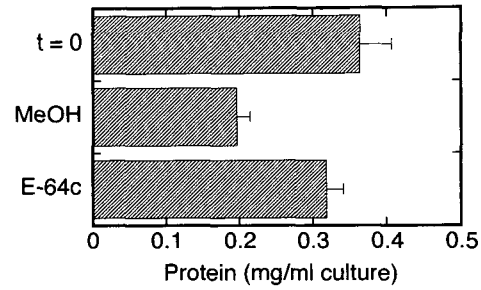

Figure 3. Inhibition of intracellular protein degradation by a Cys protease inhibitor in cultured tobacco cells. Cells were treated with 10 μM E-64c or 1% MeOH for 2 d of Suc starvation. Total proteins of the cells before (t = 0) and 2 d after Suc starvation (MeOH and E-64c) in 1 mL of culture medium were measured. Results are means ± SD (n = 5).

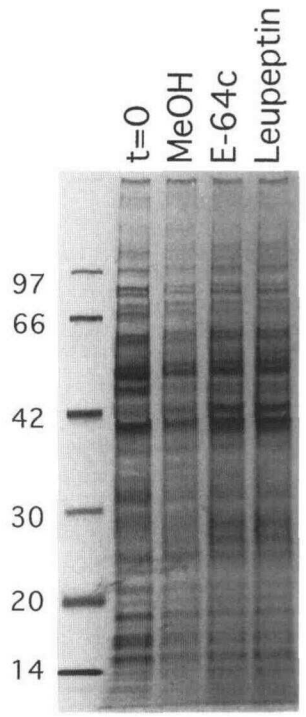

Figure 4. Nonselective degradation of intracellular proteins during Suc starvation in cultured tobacco cells. Cells were treated with 10 μM E-64c, 1% MeOH, or 10 μM leupeptin for 2 d of Suc starvation. Proteins in the homogenates of the cells before (t = 0) and 2 d after Suc starvation (MeOH, E-64c, and Leupeptin) in the same volume of culture media were analyzed by SDS-PAGE. Gels were stained with silver. Molecular masses of marker proteins were electrophoresed (shown in the left lane) and are given in kD at the left.

Spherical Bodies Accumulate with E-64c

When vacuolar protease activity is blocked genetically or with the Ser protease inhibitor PMSF in the yeast *Saccharomyces cerevisiae*, autophagic bodies (single-membrane structures containing cytoplasm) accumulate in the central vacuole (Takeshige et al., 1992). To test for the accumulation of similar structures in tobacco cells, we used a light microscope to observe the cell morphology during starvation. The cytoplasm existed not only in peripheral and perinuclear regions but also in transvacuolar strands in 4-d-old cells (i.e. cells at time 0 for all experiments done in this study) (Fig. 6A). During Suc starvation the number of transvacuolar strands decreased (Fig. 6C). After 2 d of starvation transvacuolar strands mostly disappeared, and the cytoplasm was present just beneath the plasma membrane and in the perinuclear region (Fig. 6E).

In the presence of E-64c, the transvacuolar strands disappeared in almost the same manner as that in the control

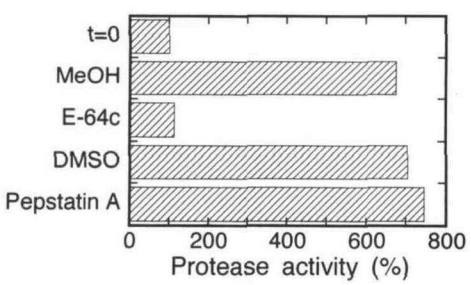

Figure 5. Inhibition of Suc starvation-induced protease activity by a Cys protease inhibitor. Cells were treated with 10 μM E-64c, 1% MeOH, 10 μM pepstatin A, or 1% DMSO for 1 d of Suc starvation. Protease activities in the homogenates of the cells before (t = 0) and 1 d after Suc starvation (MeOH, E-64c, DMSO, and Pepstatin A) were measured and are shown as relative activities per milliliter of culture medium. Values are the means of duplicate treatments in one experiment. Separate experiments were done three times, and similar results were obtained.

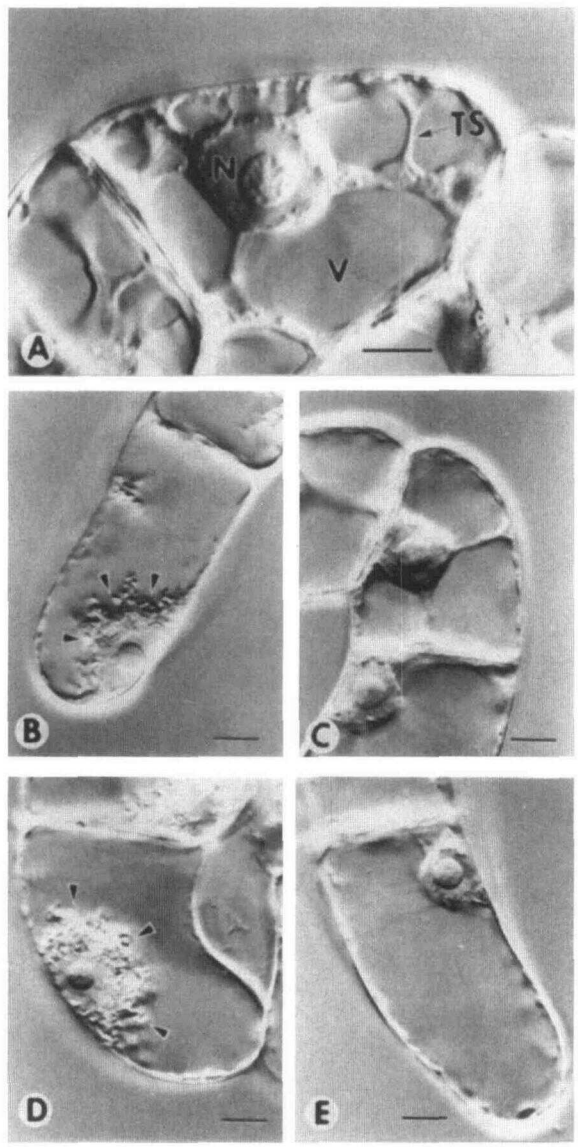

Figure 6. Morphological changes of cultured tobacco cells during Suc starvation. Cells were treated with 10 μM E-64c for 0 d (A), 1 d (B), and 2 d (D) of Suc starvation or with 1% MeOH for 1 d (C), and 2 d (E) of Suc starvation. These cells were observed on a light microscope with Nomarski optics. N, Nucleus; TS, transvacuolar strand; V, vacuole; arrowheads, spherical bodies. The bars represent 10 μm.

Table I. *Accumulation of the spherical bodies in cultured tobacco cells by various protease inhibitors*

Inhibitor	Concentration	Accumulation
	μM	
E-64c	10	++[a]
	1	+
E-64	10	++
	1	+
Leupeptin	10	++
	1	+
Antipain	100	++
	10	+
PMSF	200	−[b]
Pepstatin A	10	−

[a] Plus symbols indicate degrees of accumulation of spherical bodies in the cell. [b] Minus symbol indicates no accumulation.

cells (Fig. 6B). However, novel spherical structures appeared near the nucleus after 8 h of starvation (Fig. 6B, arrowheads). Most of these spherical bodies were less than 5 μm in diameter. The number of these spherical bodies continued to increase during 2 d of starvation (Fig. 6D, arrowheads).

Spherical Bodies Accumulate with Various Kinds of Cys Protease Inhibitors

The sperical bodies accumulated in the cells following treatment with E-64, an analog of E-64c, and also with the structurally unrelated Cys protease inhibitors leupeptin and antipain (Table I). In contrast, addition of PMSF or pepstatin A did not cause any spherical body accumulation, nor did they affect any morphological changes following Suc starvation. Thus, the inhibition of Cys protease activity is essential for the accumulation of the spherical bodies. The Cys protease inhibitors may increase the accu-

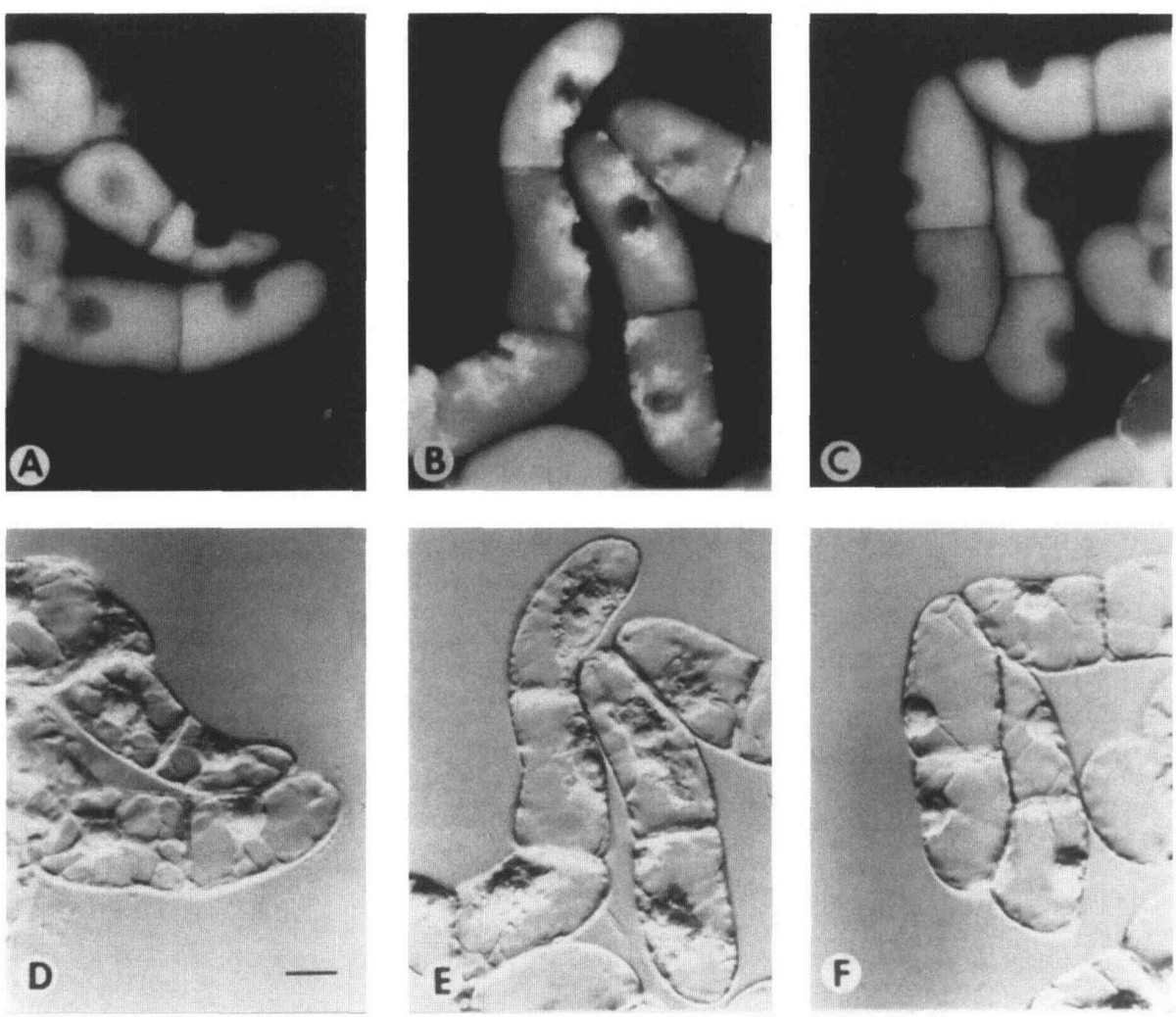

Figure 7. Staining of the spherical bodies in cultured tobacco cells with quinacrine. The cells before Suc starvation (A) and the cells treated with 10 μM E-64c (B) or with 1% MeOH (C) for 1 d of Suc starvation were stained with quinacrine. These cells were observed with a fluorescent microscope. Corresponding Nomarski images (D, E, and F) of fluorescence images (A, B, and C, respectively) are also shown. The bar represents 20 μm.

mulation of the spherical bodies by stimulating their formation or by inhibiting their disintegration.

Spherical Bodies Are Acidic Inside

We anticipated that if the spherical bodies represent autolysosomes they would be acidic inside. To check this possibility, we used acidotropic dyes (Fig. 7). Quinacrine was taken up into these bodies and into the central vacuoles (Fig. 7B). Moreover, neutral red also stained these bodies and the central vacuoles (data not shown). The protonophore carbonyl cyanide *m*-chlorophenylhydrazone quenched the staining of these bodies and the central vacuole with quinacrine (data not shown). These results show that both of these bodies and the central vacuole are acidic compartments. Quinacrine (Fig. 7B) and neutral red stained the spherical bodies more intensely than the central vacuole. Thus, the spherical bodies may be more acidic than the central vacuole, and these bodies do not directly connect to the central vacuole.

An Acid Phosphatase(s) Exists in the Spherical Bodies

To test the spherical bodies for the existence of an acid phosphatase, a marker enzyme of autolysosomes, we performed cytochemical enzyme analyses. First, we measured the change of acid phosphatase activity during Suc starvation using *p*-nitrophenylphosphate as a substrate. The phosphatase activity increased about 3-fold after 1 d of starvation, but the presence of 10 μM E-64c did not affect this activation (data not shown).

In cytochemical enzyme analysis using 1-naphthylphosphate, the spherical bodies stained red (Fig. 8A, arrowheads), which showed that an acid phosphatase(s) is localized in these bodies. In contrast, such staining was not found in control cells (Fig. 8B). The central vacuole did not stain (Fig. 8), although an acid phosphatase has been shown to be localized in vacuoles of many plant cells (Ryan and Walker-Simmons, 1983). It may be difficult to fix the enzymes in the central vacuoles by the conventional method used in this study.

Electron microscopy analysis showed that the spherical bodies are membrane-bound structures, 1 to 6 μm in diameter (Fig. 9B). These bodies contained several particles whose electron density was mostly higher than that of the cytoplasm (Fig. 9B). We could not detect these bodies in control cells (Fig. 9A). When we did a cytochemical enzyme analysis using β-glycerophosphate as a substrate, we observed precipitations of lead phosphate around these particles (Fig. 9C). This staining pattern with acid phosphatase activity is characteristic of autolysosomes shown in mammalian cells (Dunn, 1990a, 1990b) and of autophagic vacuoles detected in root meristematic cells of *Euphorbia* (Marty, 1978). Again, the enzyme cytochemical data support our hypothesis.

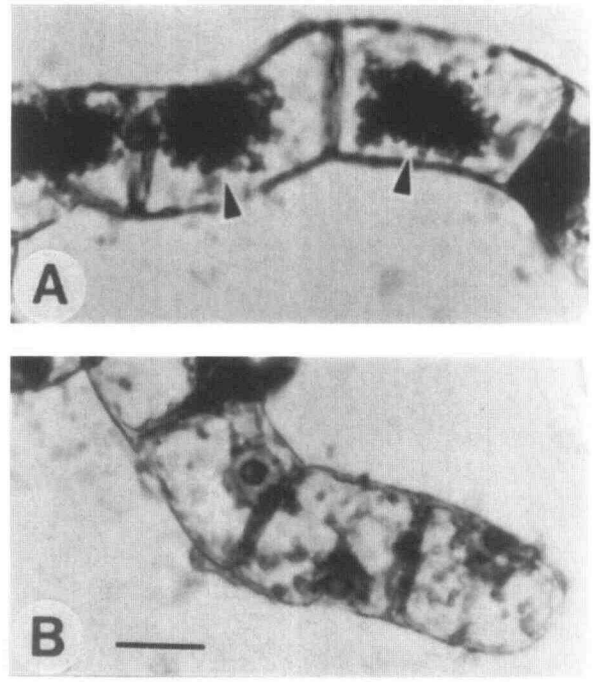

Figure 8. Staining of cultured tobacco cells for acid phosphatase activity on a light microscope. The cells treated with 10 μM E-64c (A) or with 1% MeOH (B) for 1 d of Suc starvation were fixed with 1% glutaraldehyde and 1% formaldehyde, stained for acid phosphatase activity using 1-naphthylphosphate as a substrate, and observed on a light microscope. The bar represents 20 μm. Arrowheads, Spherical bodies.

DISCUSSION

When we kept cultured tobacco (BY-2) cells under Suc starvation conditions, the total cellular protease activity increased 3- to 7-fold and net, nonselective degradation of intracellular proteins occurred. When we treated the cells with a Cys protease inhibitor such as E-64c and leupeptin, both protein degradation and the activation of the proteases were inhibited. Concomitantly, many spherical bodies accumulated in the cytosol near the nucleus. These bodies were acidic inside and contained acid phosphatase activity. Accumulation of these bodies likely occurs because the Cys protease inhibitor does not block the formation of these bodies but blocks protein degradation in them and hence their own disintegration. From all of these results we suggest that these spherical bodies are autolysosomes, where intracellular proteins are degraded during autophagy.

In yeast cells autophagy was shown to be induced by nutrient starvation (Takeshige et al., 1992; Baba et al., 1994). This process consists of the following three steps: (a) autophagosomes are formed upon nutrient starvation; (b) they fuse with a central vacuole to deliver autophagic bodies into the central vacuole; and (c) autophagic bodies are degraded in the central vacuole. When one of the vacuolar proteases, proteinase B, is inhibited by the Ser protease inhibitor PMSF, the disintegration of the autophagic bodies is inhibited, and as a result, autophagic bodies accumulate in the central vacuole. The same event occurs in mutant cells defective in proteinase B, i.e. in autophagy of yeast cells the central vacuole functions as a lysosome. The central vacuole is the only lysosomal compartment in yeast cells (Takeshige et al., 1992; Baba et al., 1994). Mature plant

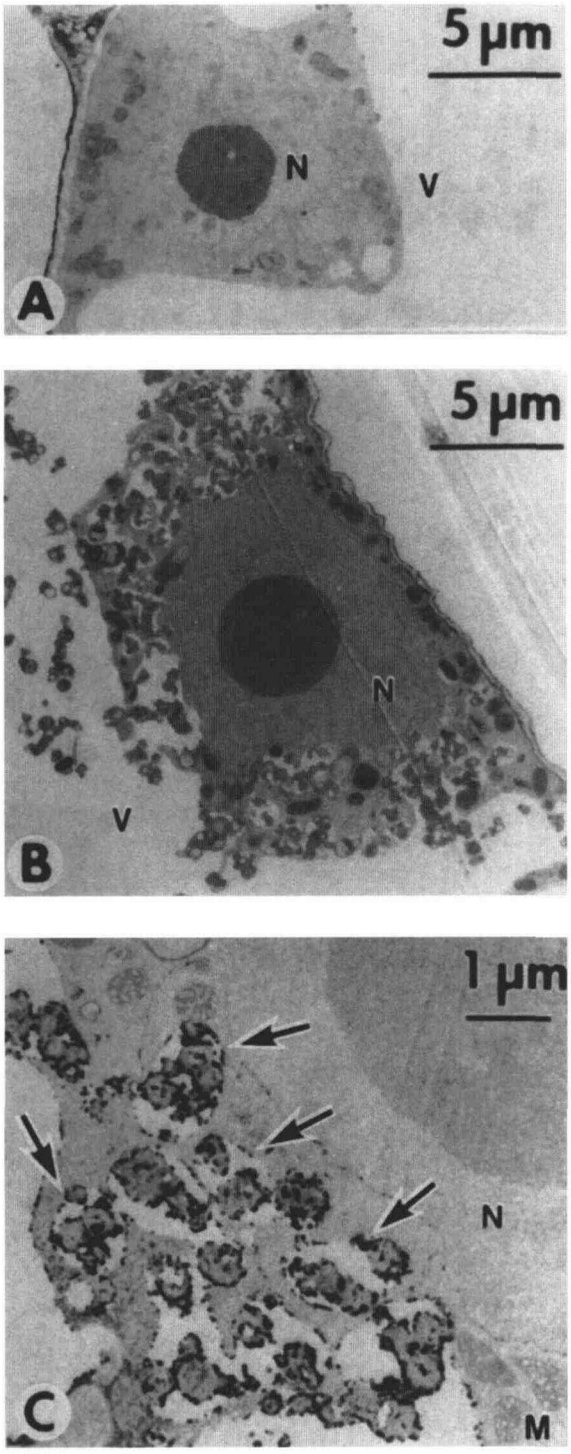

Figure 9. Cytochemical enzyme analysis of the spherical bodies in cultured tobacco cells for acid phosphatase activity. The cells treated with 10 μM E-64c (B and C) or with 1% MeOH (A) for 1 d of Suc starvation were fixed with 1% glutaraldehyde and 1% formaldehyde. Then the cells were stained for acid phosphatase activity (A and C) using β-glycerophosphate as a substrate and observed with an electron microscope. The image shown in B is from the cells treated in the same way as in C but stained without the substrate. The arrows in C indicate the spherical bodies. N, Nucleus; V, vacuole; M, mitochondrion.

cells generally have vacuoles that occupy more than 90% of the total cell volume. The vacuole in plant cells has been proposed to be a kind of lysosome, similar to the vacuole of yeast cells (Matile, 1975; Ryan and Walker-Simmons, 1983). We therefore speculated that, if vacuoles in plant cells work as lysosomes in autophagy during nutrient starvation, some portion of cytoplasmic components should accumulate in the vacuoles in the presence of protease inhibitors. However, our results suggest that there may be lytic compartments other than the central vacuole that function in autophagy.

Without a Cys protease inhibitor we could not detect the spherical bodies with either light or electron microscopy. This may imply that the spherical bodies have a short life span, which is consistent with that of autolysosomes in mammalian and yeast cells. In mammalian cells the half-life of autolysosomes is estimated to be 8 to 9 min (Mortimore and Schworer, 1977). In yeast cells the autolysosome corresponds to the central vacuole containing autophagic bodies. Without PMSF the autophagic bodies cannot be detected in wild-type cells (Takeshige et al., 1992), suggesting that the autophagic bodies are transient structures.

Based on their characteristics and by analogy to yeast cells, we suggest that the spherical bodies, which accumulate following treatment with a Cys protease inhibitor, are autolysosomes. However, we do not yet have direct evidence to link net protein degradation with the spherical bodies. Thus, we are currently determining the localization of the proteases induced by Suc starvation.

ACKNOWLEDGMENTS

We thank Dr. Takashi Mita for help with the electron microscopy, Dr. Masaharu Tamai (Taisho Pharmaceutical Co., Ltd.) for providing E-64c, and Drs. Randy Wayne, Mark Staves (Cornell University, Ithaca, NY), and Howard Damude (Salk Institute, La Jolla, CA) for critical reading of the manuscript. Y.M. also thanks Prof. Yasuhiro Miyoshi for constant encouragement.

Received November 20, 1995; accepted April 23, 1996.
Copyright Clearance Center: 0032–0889/96/111/1233/09.

LITERATURE CITED

Baba M, Takeshige K, Baba N, Ohsumi Y (1994) Ultrastructural analysis of the autophagic process in yeast: detection of autophagosomes and their characterization. J Cell Biol **124:** 903–913

Bensadoun A, Weinstein D (1976) Assay of proteins in the presence of interfering materials. Anal Biochem **70:** 241–250

Davies DD (1982) Physiological aspects of protein turnover. *In* D Boulter, B Parthier, eds, Encyclopedia of Plant Physiology, Vol 14A. Springer-Verlag, Heidelberg, Germany, pp 189–228

Dice JF (1987) Molecular determinants of protein half-lives in eukaryotic cells. FASEB J **1:** 349–357

Dunn WA (1990a) Studies on the mechanisms of autophagy: formation of the autophagic vacuole. J Cell Biol **110:** 1923–1933

Dunn WA (1990b) Studies on the mechanisms of autophagy: maturation of the autophagic vacuole. J Cell Biol **110:** 1935–1945

Goldberg AL, St John AC (1976) Intracellular protein degradation in mammalian and bacterial cells: part 2. Annu Rev Biochem **45:** 747–803

Gordon PB, Seglen PO (1989) Exogenous control of intracellular protein catabolism. *In* RJ Beynon, JS Bond, eds, Proteolytic Enzymes. IRL Press, Oxford, UK, pp 201–210

Hershko A, Ciechanover A (1982) Mechanisms of intracellular protein breakdown. Annu Rev Biochem **51:** 335–364

Huffaker RC, Peterson LW (1974) Protein turnover in plants and possible means of its regulation. Annu Rev Plant Physiol **25:** 363–392

Journet E, Bligny R, Douce R (1986) Biochemical changes during sucrose deprivation in higher plant cells. J Biol Chem **261:** 3193–3199

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem **193:** 265–275

Marty F (1978) Cytochemical studies on GERL, provacuoles, and vacuoles in root meristematic cells of *Euphorbia*. Proc Natl Acad Sci USA **75:** 852–856

Matile P (1975) The lytic compartment of plant cells. *In* Cell Biology Monographs, Vol 1. Springer-Verlag, Wien, Germany

Mortimore GE, Schworer CM (1977) Induction of autophagy by amino-acid deprivation in perfused rat liver. Nature **270:** 174–176

Noodén LD (1988) The phenomena of senescence and aging. *In* LD Noodén, AC Leopold, eds, Senescence and Aging in Plants. Academic Press, San Diego, CA, pp 1–50

Ryan CA, Walker-Simmons M (1983) Plant vacuoles. Methods Enzymol **96:** 580–589

Schworer CM, Mortimore GE (1979) Glucagon-induced autophagy and proteolysis in rat liver: mediation by selective deprivation of intracellular amimo acids. Proc Natl Acad Sci USA **76:** 3169–3173

Seglen PO (1983) Inhibitors of lysosomal function. Methods Enzymol **96:** 737–764

Takeshige K, Baba M, Tsuboi S, Noda T, Ohsumi Y (1992) Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction. J Cell Biol **119:** 301–311

Twining SS (1984) Fluorescein isothiocyanate-labeled casein assay for proteolytic enzymes. Anal Biochem **143:** 30–34

Vierstra RD (1993) Protein degradation in plants. Annu Rev Plant Physiol Plant Mol Biol **44:** 385–410