Autophagy is a universal process by which cellular proteins are degraded via a lysosomal/vacuolar system. Depending on the extracellular nutrient conditions, the rate of autophagic protein degradation fluctuates between 1–1.5% and 4–5% of total cell proteins per hour (1, 2). There are two pathways of autophagy, microautophagy and macroautophagy (for reviews, see Refs. 3 and 4). In microautophagy, relatively small portions of the cytoplasm are directly enclosed by invaginating lysosomal membranes. The rate of autophagic degradation of bulk cell constituents by the lysosome/vacuole system occurs via macroautophagy. In the initial step of macroautophagy, various cytosolic proteins, as well as cytoplasmic organelles such as mitochondria, endoplasmic reticulum (ER), and peroxisomes, are sequestered in the lumen of double-membrane autophagosomes. Autophagosomes then fuse with endosomes or lysosomes to become mature, single-membrane autolysosomes. Acidification of the lumen and acquisition of lysosomal hydrolytic enzymes enable this specialized membrane system to degrade sequestered cytoplasmic components.

The origin of the autophagosomal membrane is a subject of controversy. Extensive morphological analyses by Dunn (5) indicated that the autophagosomal membrane derives from the rough ER. However, the post-Golgi membrane, as well as a unique de novo synthesized membrane, the phagophore, have also been proposed as sources (6, 7). In recent morphological studies on yeast autophagy (8, 9), autophagosomes were found to have features distinct from those of other pre-existing cell membranes. This appears to support the notion that the membrane may have a unique origin. Understanding the molecular organization of the autophagosomal membrane is important for understanding the mechanism of autophagy at the membrane level, since various key molecules involved in or necessary for the formation and fusion of autophagosomes are likely to exist on the autophagosomal membrane. In order to characterize the autophagosomal membrane, it is necessary to isolate autophagosomes. However, autophagosome matura- tion proceeds so quickly that it is very difficult to isolate auto-

* This work was supported by Grant-in-aid for Scientific Research 09680629 and Grant-in-aid for Scientific Research on Priority Areas (Intracellular Proteolysis) 08278103 from the Ministry of Education, Science, Sports and Culture of Japan and the Japan Science Promotion Fund from the Japan Private School Promotion Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom all correspondence should be addressed: Dept. of Biochemistry, Juntendo University School of Medicine, Bldg. 9, Rm. 913, Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. Tel.: 813-5802-1031; Fax: 813-5802-5889; E-mail: kominami@med.juntendo.ac.jp.

† This paper is available on line at http://www.jbc.org

© 1999 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.

Autolysosomal Membrane-associated Betaine Homocysteine Methyltransferase

LIMITED DEGRADATION FRAGMENT OF A SEQUESTERED CYTOSOLIC ENZYME MONITORING AUTOPHAGY*

(Received for publication, January 11, 1999, and in revised form, March 6, 1999)

Takashi Ueno‡, Kazumi Ishidoh‡, Reiko Mineki‡, Isei Tanida‡, Kimie Murayama§, Motoni Kadowaki‡, and Eiki Kominami¶‡

From the ‡Department of Biochemistry and §Central Laboratory for Medical Sciences, Juntendo University School of Medicine, Hongo, Bunkyo-ku, Tokyo 113-8421 and the ¶Department of Applied Biochemistry, Faculty of Agriculture, Niigata University, Igarashi, Niigata 950-21, Japan

We compared the membrane proteins of autolysosomes isolated from leupeptin-administered rat liver with those of lysosomes. In addition to many polypeptides common to the two membranes, the autolysosomal membranes were found to be more enriched in endoplasmic reticulum luminal proteins (protein-disulfide isomerase, calreticulin, ER60, BiP) and endosome/Golgi markers (cation-independent mannose 6-phosphate receptor, transferrin receptor, Golgi 58-kDa protein) than lysosomal membranes. The autolysosomal membrane proteins include three polypeptides (44, 35, and 32 kDa) whose amino-terminal sequences have not yet been reported. Combining immunoblotting and reverse transcriptase-polymerase chain reaction analyses, we identified the 44-kDa peptide as the intact subunit of betaine homocysteine methyltransferase and the 35- and 32-kDa peptides as two proteolytic fragments. Pronase digestion of autolysosomes revealed that the 44-kDa and 32-kDa peptides are present in the lumen, whereas the 35-kDa peptide is not. In primary hepatocyte cultures, the starvation-induced accumulation of the 32-kDa peptide occurs in the presence of E64d, showing that the 32-kDa peptide is formed from the sequestered 44-kDa peptide during autophagy. The accumulation is induced by rapamycin but completely inhibited by wortmannin, 3-methyladenine, and bafilomycin. Thus, detection of the 32-kDa peptide by immunoblotting can be used as a streamlined assay for monitoring autophagy.

Autophagy*
phagosomes of sufficient purity and in quantities suitable for biochemical analyses. Therefore, we decided to take an indirect approach. Autolysosomes isolated from leupeptin-administered rat liver have some advantages. First, they can be easily purified by Percoll-gradient centrifugation and obtained in quantity (10, 11). Second, effective inhibition of lysosomal proteolysis by leupeptin keeps many of the sequestered cytoplasmic proteins apparently active or undegraded (12, 13). As a result, it is expected that some membrane components characteristic of autophagosomes may also be preserved on autolysosomal membranes. In a previous study (13), we found that isolated autolysosomal membranes possess two ER membrane proteins, cytochrome P450 and NADPH-cytochrome P450 reductase. These results are consistent with those of Dunn (5) in showing that autophagosomes originate from the ER. It is interesting to clarify other components in isolated autolysosomal membranes, especially in relation to autophagosomes. In this study, we systematically analyzed membrane proteins in isolated autolysosomes by two-dimensional gel electrophoresis and compared the results with those of lysosomes isolated from dextran-loaded rat liver.

**EXPERIMENTAL PROCEDURES**

**Animals**—Male Wistar rats (250–300 g) were maintained in an environmentally controlled room (lights on 6:00 to 20:00) for at least 2 weeks before experiments. All rats were fed a standard pelleted laboratory diet and tap water ad libitum during this period. For all experiments, the rats were starved for 12–18 h before use.

**Primary Culture of Rat Hepatocytes**—Hepatocytes were isolated from 18-h-starved male Wistar rats by a collagenase perfusion procedure (14). The hepatocytes were seeded at a density of 10^3 cells/0.2 ml/cm^2 and cultured in Williams E medium supplemented with 10% fetal calf serum (Williams E/10% FCS).

**Antibodies and Reagents**—Protein was determined by the BCA protein assay following the manufacturer's protocol (Pierce). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (15). Immunoblot analyses were performed according to the method of Towbin et al. (16) except that 2,4-dichloro-1-naphthol (17) or an ECL Western blot detection kit (Amersham Pharmacia Biotech) was used as the substrate for the peroxidase reaction. Antibodies against a synthetic peptide corresponding to a sequence in rat 7 (residues 175–191) was produced in rabbits as described by Chavrier et al. (20). Antibody to cation-independent mannos 6-phosphate receptor (Cl-M6PR) was produced in rabbits as described (21). Commercially available antibodies were purchased from the following sources: antibodies against rab 5A, trimeric GTP-binding protein subunits Gαs, Gαi2, and Gβγ from Santa Cruz Biochemicals; antibody to Golgi 58-kDa protein from Sigma. A monoclonal antibody against transferrin receptor was produced as described (19). Antibodies were affinity-purified on immo- bilized peptide-Sepharose columns. Antibody against a synthetic peptide was affinity-purified on immobilized peptide-Sepharose columns. Antibody against a synthetic peptide was affinity-purified on immobilized peptide-Sepharose columns.

**RT-PCR**—Total RNA was isolated from rat liver using guanidinium thiocyanate/TeCl ultracentrifugation (26). Poly(A) RNA was isolated from total RNA of oligo(dT)-cellulose according to the manufacturer's protocol.

Degenerated primers deduced from the amino acid sequences at the protein level were synthesized: CCNATHGCGNNAARAGGC designated as p44–1, AAYGGCNGGARGRTGNATTGGH as p44–2, GCNACITCGCNRCTCNCCYCTCRT as p32–1R, and GNCCTCTRTITNA CYTTYTGNC as p32–2R. RT-PCR using the primer set p44–1 and p32–1R from rat liver poly(A) RNA was accomplished at an annealing temperature of 55 °C by an RT-PCR kit (Toyobo, Tokyo, Japan). An aliquot of sample was further subjected to nested PCR with the primer set p44–2 and p32–2R at an annealing temperature of 57 °C. After subcloning into pCRII vector (Invitrogen), some of the insert-positive clones were sequenced. Two clones sequenced from each strain were sequenced by the dideoxynucleotide chain-termination method. The sequenced DNA was sequenced after digestion with HindIII. The DNA sequence was compared with the amino acid sequence determined at the protein level, and two clones were identified as the cDNA for p44.

**Two-dimensional Gel Electrophoresis and Protein Sequence Analysis**—Protein separation by two-dimensional gel electrophoresis was performed according to the method of O'Farrell (24) with slight modifications. For isoelectric focusing, the gels were polymerized in Pyrex tubes (inner diameter, 3 mm; height, 12 cm) to give a gel height of 9.5 cm. Gels containing 4% acrylamide, 0.22% N,N'-methylene bis(acrylamide), 9.2% urea, 2.5% Nonidet P-40, 1.5% octyl glucoside, 1.2% Amphoteric (pH 5–8), 0.4% Amphoteric (pH 3.5–5.9), and 0.4% Amphoteric (pH 3.5–9.5) were prepared for analyses at acidic pH (pH 5–8). The electrode solutions used were 20 mM NaOH (cathode) and 0.2% phosphoric acid (anode). Gels containing 4% acrylamide, 0.22% N,N'-methylene bis(acrylamide), 9.2% urea, 2.5% Nonidet P-40, 0.5% n-dodecyl-β-D-maltoside, 1.6% Amphoteric (pH 7–9), and 0.4% Amphoteric (pH 3.5–9.5) were prepared for analyses at alkaline pH (pH 6–8). The electrode solutions used were 0.1 M NaOH (cathode) and 10 mM phosphoric acid (anode). All samples applied to a gel contained the same amount of protein. SDS-PAGE in the second dimension was performed using 5–15% linear gradient gels. After SDS-PAGE, the gels were fixed in 10% methanol containing 10% acetic acid, and subsequently silver-stained.

For amino-terminal amino acid sequence analysis, the proteins separated by two-dimensional gel electrophoresis were electrothermally transferred onto nitrocellulose membranes (Immobilon P™, Millipore Corp.) using 10 mM Caps (pH 11) containing 20% methanol as the electrode buffer. The membrane was stained with Coomassie Brilliant Blue, destained with 10% acetic acid containing 50% methanol (destaining solution), and air-dried. Pieces containing individual spots were cut out and soaked in destaining solution, washed several times with 10% acetic acid, and air-dried. The amino-terminal amino acid sequences of proteins fixed on Immobilon P™ membranes were determined with a protein sequence analyzer (Hewlett Packard, model G1005A).

**GTP Blot Analysis**—GTP blotting assays were carried out according to the method of Huber et al. (25). Autolysosomal and lysosomal membrane proteins resolved by isoelectric focusing and subsequent SDS-PAGE in the second dimension (15% gels), as described in the previous section, were electrothermally transferred onto nitrocellulose membranes (Advantec Toyo, Tokyo, Japan). The nitrocellulose membrane sheets were incubated with [α-32P]GTP (10 μCi) in the presence of 4 μM ATP at room temperature for 2 h, washed with 50 mM NaH2PO4 (pH 7.5) containing 10 μM MgCl2, 0.2% Tween 20, 4 μM ATP, and 2 mM dithioerythritol, and then air-dried. The incorporation of radioactive GTP was detected by autoradiography. Identical sheets were used for immunoblotting to identify GTP-binding proteins after washing the sheets with 1% acetic acid to remove membrane-bound GTP.

**Pronase Treatment of Autolysosomes**—Freshly isolated autolysosomes were incubated at 0 °C in medium (1 ml) containing 5 mM Tes (pH 7.5), 0.3 μM sucrose, and Pronase E (0.2–0.8 mg/ml) in the presence or absence of 0.2% Triton X-100. The reaction was stopped by adding an equal volume of 10% trichloroacetic acid. The pellets were collected by centrifugation at 5,000 × g for 5 min at 4 °C, washed once with 0.1 M NaCl, neutralized by 0.5 M Na2CO3, and vigorously shaken on a microtube shaker for 5 min. The samples were then solubilized with SDS-PAGE sample buffer, boiled in a water bath for 3 min, and electrophoresed in 10% SDS-polyacrylamide gels.

**RT-PCR**—Total RNA extracted from rat liver was subjected to guanidinium thiocyanate/TeCl ultracentrifugation (26). Poly(A) RNA was isolated from total RNA of oligo(dT)-cellulose according to the manufacturer's protocol.

Degenerated primers deduced from the amino acid sequences at the protein level were synthesized: CCNATHGCGNNAARAGGC designated as p44–1, AAYGGCNGGARGRTGNATTGGH as p44–2, GCNACITCGCNRCTCNCCYCTCRT as p32–1R, and GNCCTCTRTITNA CYTTYTGNC as p32–2R. RT-PCR using the primer set p44–1 and p32–1R from rat liver poly(A) RNA was accomplished at an annealing temperature of 55 °C by an RT-PCR kit (Toyobo, Tokyo, Japan). An aliquot of sample was further subjected to nested PCR with the primer set p44–2 and p32–2R at an annealing temperature of 57 °C. After subcloning into pCRII vector (Invitrogen), some of the insert-positive clones were sequenced. Two clones sequenced from each strain were sequenced by the dideoxynucleotide chain-termination method. The sequenced DNA was sequenced after digestion with HindIII. The DNA sequence was compared with the amino acid sequence determined at the protein level, and two clones were identified as the cDNA for p44. To isolate the 5′ region further upstream of the cDNA, we carried out the RACE reaction using nucleotide sequence primers including CATGACGTTGGATCCAGCTCTG as Up-2 for 5 min and CATGACGTTGGATCCAGCTCTG as Up-1 and CTGTCACAGGGTGGATCCAGCTCTG as Up-2 for 5–RACE at an anneal-
ing temperature of 57°C with rat liver poly(A) \(^+\) RNA with a Marathon™ cDNA amplification kit (CLONTECH). After subcloning into pCRII vector, the nucleotide sequences were determined by the dye-primer method in an Applied 373A DNA sequencer.

**RESULTS**

Identification of Autolysosomal Membrane Polypeptides Separated by Two-dimensional Gel Electrophoresis—In order to identify as many polypeptides present in greater quantity in autolysosomal membranes than in lysosomal membranes as possible, we performed preparative two-dimensional gel electrophoretic analyses at different pH ranges to allow us to identify major membrane polypeptides directly by amino acid sequence determination. It should also be noted that the carbonate treatment of the membranes carried out in the previous study (13) was omitted so as not to overlook peripheral membrane proteins. Fig. 1 shows silver-stained protein spots separated at pH 4–7 (Fig. 1, A and B) and pH 6–8 (Fig. 1, C and D). Basically, autolysosomal membranes and lysosomal membranes closely resemble one another, indicating that autolysosomes from leupeptin-treated liver have reached a substantial level of maturation. However, some spots appear to be more enriched, or present only in autolysosomal membranes (Fig. 1, A and C). The spots (a–u) shown by arrows (Fig. 1, A and C) were reproducibly found among different preparations, and because almost all of these spots could be stained by Coomassie Brilliant Blue after electrophoretic transfer to PVDF membranes, we attempted to identify the polypeptides by protein sequence analysis.

The amino-terminal sequences of these polypeptides are summarized in Table I. Unambiguous sequences were not obtained for 9 of the 21 polypeptides analyzed, possibly due to blocked amino termini (spots d, p, and t) or insufficient amounts of amino acids detected (spots g, h, i, j, l, and n). Several major polypeptides separated by isoelectric focusing in the acidic pH range (Fig. 1A) were identified as ER luminal proteins, including protein-disulfide isomerase, calreticulin, ER60 protease, and BiP. In contrast, no polypeptides were identified as being of post-Golgi membrane origin. There are five polypeptides (spots m, o, q, r, and u) whose amino-terminal sequences have not yet been reported. No further analysis was made of spot m, because the quantity obtained was too small; information on the sequence beyond residue 10 is not yet available. The remaining four components were identified as betaine homocysteine methyltransferase and its partially degraded fragments, as described below.

Identification of Golgi/Endosome Membrane Markers, Rab GTP-binding Proteins, and Trimeric GTP-binding Protein Subunit as—As clearly seen in the previous section, there are no major Golgi/endosome components in amounts sufficient for identification by amino acid sequence determination. The immunoblots shown in Fig. 2A show that autolysosomal but not lysosomal membranes possess three Golgi/endosome markers, transferrin receptor, Cl-M6PR, and Golgi 58-kDa protein. As transferrin receptor and Cl-M6PR are early and late endosome markers, respectively, we took notice of the rab GTP-binding proteins, because it has been found that distinctive rab GTP-binding proteins characterize distinct compartments of intracellular membranes (27). The GTP blots indicate that both autolysosomal and lysosomal membranes have almost identical sets of rab GTP-binding proteins, except that rab 5A, identified by immunoblot, is more abundant in lysosomal membranes than in autolysosomal membranes (Fig. 2B). Although no incorporation of \(^{32P}\)GTP into rab7 is seen in the GTP blots, it is detected by immunoblot in both autolysosomal and lysosomal membranes (Fig. 2C).

The roles of trimeric GTP-binding proteins in ER-Golgi transport, homotyp membrane fusion of lysosomes, and in the maintenance of Golgi structures have been reported recently (28–30). It has also been reported that trimeric GTP-binding protein subunits are associated with Golgi membranes and the trans-Golgi network (31, 32). We confirmed by immunoblotting that the trimeric GTP-binding protein subunits, G\(_{\alpha}\) and G\(_{\beta}\), are associated with isolated autolysosomal and lysosomal membranes (Fig. 2D). Both G\(_{\alpha}\) and G\(_{\alpha}\) appear to be evenly distributed in the two membranes, whereas G\(_{\alpha}\) seems more abundant in autolysosomal membranes.

Identification of Three Major Polypeptides Associated with Autolysosomal Membranes as Betaine Homocysteine Methyltransferase—As mentioned in the previous section, there are four major polypeptides (spots o, q, r, and u in Fig. 1) in autolysosomal membranes whose amino-terminal sequences have not yet been reported. Based on the apparent molecular sizes determined by mobility in SDS-PAGE, we designate spot u as p44 and spot q as p35. Spot o and spot r have identical sequences despite their apparently different PI values. We therefore designate the two components as p32 without further discrimination. We first attempted to determine as many amino acid residues as possible toward the carboxyl terminus by protein sequencing; the data are summarized in Table II. It appears obvious that p32 must be processed from p35, because, except for the first four amino acid residues, Y, V, A, and E, the two polypeptides have identical sequences. Immunoblot analyses using antibodies raised against synthetic decapeptides corresponding to 10 amino-terminal residues further support the possibility that both p35 and p32 derive from a common precursor form, p44. As Fig. 3 shows, the antibody to p32 (p-p32–10R) recognizes all three polypeptides and the p35 antibody (p-p35–10R) reacted with both p35 and p44 (Fig. 3, A and B).

In order to confirm the above possibility further, we carried out RT-PCR using degenerated primer sets deduced from the amino acid sequences of p44 and p32 as described under “Experimental Procedures.” Fragments of about 250 base pairs were amplified and sequenced after subcloning into pCRII vector. The nucleotide sequences were 258 base pairs in length, and the deduced amino acid sequences contained the amino acid sequences of both p44 and p32 (Fig. 4A). To isolate a region on the 5‘-upstream side of the cDNA for the precursor protein, 5‘-RACE was carried out using the specific primers, Up-1 and Up-2. One clone isolated from the 5′-RACE reaction when subcloned into pCRII vector encodes the 5′-end of the precursor protein (Fig. 4B). The nucleotide sequence of this cDNA, which includes a region that overlaps with the first RT-PCR product, was subjected to a nucleotide sequence homology search using the computer package, BLAST. It was found that the nucleotide sequence of the cDNA for human betaine homocysteine methyltransferase (BHMT) (33) shows 86% identity at the nucleotide sequence level and 93% identity at the deduced amino acid sequence level to that of the cDNA clone isolated in our study. Thus, p35 and p32 have been identified as the proteolytic products of p44, i.e. rat BHMT. (Recently, the full-length cDNA for rat BHMT was registered in GenBank with accession number U96133.)

Sequestration and Degradation of BHMT during Autophagy—BHMT is a major cytosolic protein accounting for nearly 1.6% of the total cytosolic protein in the liver (33). We therefore reasoned that BHMT is originally sequestered as a cytoplasmic component, the substrate of autophagy, into autophagosomes, and subsequently degraded to p35 and p32 by some steps during the maturation process from autophagosomes to autolysosomes. To examine this possibility further, we attempted to determine if autolysosomal BHMT and the two fragments truly exist in the lumen. Since antibodies raised against synthetic
peptides corresponding to the 10 amino-terminal residues of p35 and p32 (α-p35–10R and α-p32–10R) also recognized p44, we first tried to prepare peptide antibodies that do not react with p44 but more specifically recognize p35 or p32 to avoid complications to the experimental data. As the results of immunoblotting show (Fig. 5A, lane 2), antibody raised against the five amino-terminal residues of p35 (α-p35–5R) was found to react only with p35. Likewise, antibody raised against the five amino-terminal residues of p32 (α-p32–5R) recognized p32 but not p35 or p44 (Fig. 5A, lane 3). Using these newly prepared antibodies together with an anti-p44 antibody (α-p44–10R), we next analyzed the distribution of p44, p35, and p32 in autolysosomal subfractions. As shown in Fig. 5B, both p44 and p32 exist in autolysosomal membranes and the sediment fraction, which consists mainly of sequestered cytoplasmic components, whereas p35 exists in autolysosomal membranes but not in the sediment. Furthermore, Pronase treatment of isolated autolysosomes confirmed that p44 and p32 are present in the autolysosomal lumen but that p35 is associated with the outer surface of autolysosomes (Fig. 5C). Incubation of autolysosomes with Pronase at 0 °C for 10 min resulted in the complete digestion of p35 irrespective of the presence or absence of Triton X-100 (Fig. 5C, lanes 4–6). Under these conditions, p44 was degraded in the presence of Triton X-100 but resistant to digestion in its absence (Fig. 5C, lanes 1–3). p32 could not be degraded by Pronase regardless of the presence of added Triton X-100 (Fig. 5C, lanes 7–9). This suggests that most intra-autolysosomal p32 exists in denatured protein aggregates (sediment fraction) together with other sequestered proteins. From these data, we conclude that BHMT (p44) is sequestered into autophagosomes and subsequently degraded upon maturation of the autophagosomes to autolysosomes. p32, a limit-digested fragment of p44, accumulates as a result of the cessation of autophagic proteolysis in the presence of leupeptin/E64c; otherwise, it would be degraded rapidly and completely. In fact, p32 could not be detected in any subcellular fraction, even under starva-

### Table I

| Spot | Sequence | Identity |
|------|----------|----------|
| a    | DDAYFKEQF | Calreticulin |
| b    | DALEEEDWL | Protein-disulfide isomerase |
| c    | EEEDKEDVG | BiP (GRP78) |
| d    | Undetermined | Unknown |
| e    | SDVLELTEN | ER-60 protease |
| f    | XVLELTEN | ER-60 protease |
| g    | Undetermined | Unknown |
| h    | Undetermined | Unknown |
| i    | Undetermined | Unknown |
| j    | Undetermined | Unknown |
| k    | YPSMWRKK | Cathepsin H |
| l    | Undetermined | Unknown |
| m    | SARANGQAYE | Unidentified |
| n    | Undetermined | Unknown |
| o    | KISQKVKNEA | Unidentified |
| p    | Undetermined | Unknown |
| q    | YVAEIKOSQK | Unidentified |
| r    | KISQKVKNEA | Unidentified |
| s    | XHSLPDLFYD | Superoxide dismutase |
| t    | Undetermined | Unknown |
| t    | APIAKKAAK | Unidentified |

**Fig. 1.** Polypeptide composition of autolysosomal membranes and lysosomal membranes separated by two-dimensional gel electrophoresis. Eighty micrograms of protein from autolysosomal (A and C) and lysosomal (B and D) membranes were separated by isoelectric focusing performed at either acidic pH (A and B) or alkaline pH (C and D). SDS-PAGE was carried out in the second dimension using linear gradient (5–15%) SDS-polyacrylamide gels. After electrophoresis, the gels were silver-stained. The polypeptides that were present solely or more abundantly in autolysosomal membranes are indicated by arrows with lowercase letters (a–u). The positions of molecular mass markers (in kDa) are shown on the right.
**Autolysosomal Membrane-associated Proteins**

**Fig. 2.** Golgi/endosome marker proteins, rab GTP-binding proteins, and α subunits of trimeric GTP-binding proteins in autolysosomal and lysosomal membranes. A, 100 µg of protein from autolysosomal (AL) and lysosomal (L) membranes were electrophoresed on an SDS-polyacrylamide gel (7.5%). The separated proteins were electrotransferred onto Millipore membrane filters (GV, 0.22-µm pore size) and analyzed by immunoblot using monoclonal anti-transferrin receptor antibody (TR), polyclonal anti-CI-M6PR antibody (CI-M6PR), and anti-Golgli 58-kDa protein antibody (G58K). B, 80 µg of protein from autolysosomal (AL) and lysosomal (L) membranes were separated by isoelectric focusing at alkaline pH as described under “Experimental Procedures” and subsequently electrophoresed on 15% acrylamide gels. The separated proteins were electrotransferred onto a nitrocellulose membrane filter, and the membrane filter was incubated with [α-32P]GTP. The radioactive spots were visualized by autoradiography. For details, see “Experimental Procedures.” The membrane filter was then used for the identification of rab 5A and rab 7 by immunoblot. The arrowhead indicates the radioactive spot identified as rab 5A. C. Immunoblot showing rab 7 on the same membrane filter previously used for GTP blot analysis. D, equal amounts of protein (100 µg) from autolysosomal membranes (AL) and lysosomal membranes (L) were electrophoresed in 10% SDS-polyacrylamide gels. Separated proteins were transferred onto a Millipore membrane filter (GV, 0.22-µm pore size). The membrane filter strips were then immunoblotted using anti-Gα antibody, an antibody recognizing both GαS and Gαi3, and anti-Gαi3-specific antibody.

**DISCUSSION**

As shown by two-dimensional gel electrophoresis, we were able to use lysosomal membranes isolated from dextran-loaded liver as a reference to identify autolysosomal polypeptides of nonlysosomal origin. Most of these polypeptides derive from either the ER or endosome/Golgi compartment, i.e. from preexisting membranes. The question, then, is can these nonlysosomal polypeptides be somehow related to the origin and maturation of autophagosomal membrane?

The identification of major autolysosomal membrane-associated polypeptides as ER lumenal proteins (protein-disulfide isomerase, calreticulin, ER60 protease, and BIP) is apparently consistent with the data of Dunn (5), indicating that ER luminal content exists in the outer limiting membranes of autophagosomes. Together with the cytochrome P450 and NADH-cytochrome P450 reductase detected in our previous study (13), these ER markers in the autolysosomal membrane may be explained as surviving autophagosomal membrane components. However, the existence of ER components on autophagosomal membranes has been a subject of dispute. In immunoelectron microscopic studies, Masaki et al. (39) and Yamamoto et al. (40) reported that cytochrome P450 does not exist on the surface membranes of autolysosomes and autophagosomes, but rather in the lumen as a substrate to be degraded via autophagy. The different antibodies used and the different stages of maturation of the autophagosomal and/or autolysosomal particles observed may partially explain the discrepancy in the data among these laboratories. Moreover, an extreme possibility may also be considered; a specific region of the ER membrane may be used as a source of surface limiting membranes as reported by Dunn (5) on the one hand, and bulk ER membranes may be sequestered in autophagosomes on the other. In order to obtain a firmer conclusion about this problem, a thorough morphological examination using antibodies raised against as many ER components as possible should be performed on autophagosomes/autolysosomes at various stages of autophagy.

There were no major polypeptides of endosomal/Golgi origin in amounts sufficient for amino acid sequence analysis. Thus, we could not obtain evidence that autolysosomal membranes

---

**p32 as an Endogenous Marker of Autolysosome Maturation**

Detection of cellular p32 using α-p32–10R and α-p32–5R in the presence of leupeptin or E64 will thus become a useful means of identifying autolysosomes. The data shown in Fig. 6 indicates the radioactive spot identified as p32. The 26-kDa protein could be detected by both α-p32–10R and α-p32–5R, and was also found in autolysosomes (Figs. 3 and 5). However, we could not detect the 26-kDa protein as an obvious spot in two dimensional gel electrophoresis (Fig. 1). It is also noted that p35 could not be detected in immunoblots using α-p35–5R (data not shown). In the experiments shown in Fig. 6B, the effects of various inhibitors and an activator of autophagy on the accumulation of p32 were investigated. The accumulation of p32 was completely blocked by 3-methyladenine (34), wortmannin (35), and bafilomycin (36), known inhibitors of autophagy (Fig. 6B, lanes 7–9). In contrast, rapamycin, which stimulates hepatic autophagy by inhibiting S6 kinase activity (37, 38), induced p32 accumulation even when the hepatocytes were incubated with Williams E/10% FCS (Fig. 6B, lanes 4–6). Thus, the E64d-dependent accumulation of p32 in cultured hepatocytes corresponds well to the activity of hepatic autophagy at the time when E64d inhibits lysosomal cysteine proteinasises under starvation conditions.

---

**Autolysosomal Membrane-associated Proteins**

**Fig. 2.** Golgi/endosome marker proteins, rab GTP-binding proteins, and α subunits of trimeric GTP-binding proteins in autolysosomal and lysosomal membranes. A, 100 µg of protein from autolysosomal (AL) and lysosomal (L) membranes were electrophoresed on an SDS-polyacrylamide gel (7.5%). The separated proteins were electrotransferred onto Millipore membrane filters (GV, 0.22-µm pore size) and analyzed by immunoblot using monoclonal anti-transferrin receptor antibody (TR), polyclonal anti-CI-M6PR antibody (CI-M6PR), and anti-Golgli 58-kDa protein antibody (G58K). B, 80 µg of protein from autolysosomal (AL) and lysosomal (L) membranes were separated by isoelectric focusing at alkaline pH as described under “Experimental Procedures” and subsequently electrophoresed on 15% acrylamide gels. The separated proteins were electrotransferred onto a nitrocellulose membrane filter, and the membrane filter was incubated with [α-32P]GTP. The radioactive spots were visualized by autoradiography. For details, see “Experimental Procedures.” The membrane filter was then used for the identification of rab 5A and rab 7 by immunoblot. The arrowhead indicates the radioactive spot identified as rab 5A. C. Immunoblot showing rab 7 on the same membrane filter previously used for GTP blot analysis. D, equal amounts of protein (100 µg) from autolysosomal membranes (AL) and lysosomal membranes (L) were electrophoresed in 10% SDS-polyacrylamide gels. Separated proteins were transferred onto a Millipore membrane filter (GV, 0.22-µm pore size). The membrane filter strips were then immunoblotted using anti-Gα antibody, an antibody recognizing both GαS and Gαi3, and anti-Gαi3-specific antibody.

**DISCUSSION**

As shown by two-dimensional gel electrophoresis, we were able to use lysosomal membranes isolated from dextran-loaded liver as a reference to identify autolysosomal polypeptides of nonlysosomal origin. Most of these polypeptides derive from either the ER or endosome/Golgi compartment, i.e. from preexisting membranes. The question, then, is can these nonlysosomal polypeptides be somehow related to the origin and maturation of autophagosomal membrane?

The identification of major autolysosomal membrane-associated polypeptides as ER lumenal proteins (protein-disulfide isomerase, calreticulin, ER60 protease, and BIP) is apparently consistent with the data of Dunn (5), indicating that ER luminal content exists in the outer limiting membranes of autophagosomes. Together with the cytochrome P450 and NADH-cytochrome P450 reductase detected in our previous study (13), these ER markers in the autolysosomal membrane may be explained as surviving autophagosomal membrane components. However, the existence of ER components on autophagosomal membranes has been a subject of dispute. In immunoelectron microscopic studies, Masaki et al. (39) and Yamamoto et al. (40) reported that cytochrome P450 does not exist on the surface membranes of autolysosomes and autophagosomes, but rather in the lumen as a substrate to be degraded via autophagy. The different antibodies used and the different stages of maturation of the autophagosomal and/or autolysosomal particles observed may partially explain the discrepancy in the data among these laboratories. Moreover, an extreme possibility may also be considered; a specific region of the ER membrane may be used as a source of surface limiting membranes as reported by Dunn (5) on the one hand, and bulk ER membranes may be sequestered in autophagosomes on the other. In order to obtain a firmer conclusion about this problem, a thorough morphological examination using antibodies raised against as many ER components as possible should be performed on autophagosomes/autolysosomes at various stages of autophagy.

There were no major polypeptides of endosomal/Golgi origin in amounts sufficient for amino acid sequence analysis. Thus, we could not obtain evidence that autolysosomal membranes
are structurally related to the Golgi/endosome compartment. The existence of endosomal markers, such as transferrin receptor, CI-M6PR, and several rab GTP-binding proteins, should rather be evaluated with respect to the convergence of autophagic and endocytic pathways as reported by Gordon and Seglen (41) and Dunn (42). Our data are consistent with a more recent report by Berg et al. (43), showing that amphisomes (prelysosomal autophagic/endosomal vacuoles) possess both early and late endosome markers.

The association of transferrin receptor may reflect the possibility that the receptor is also brought in from recycling endosomes that have been found to be distinct from sorting early endosomes (44). In contrast, rab 5, another early endosome marker, is more enriched in lysosomal membranes than in autolysosomal membranes. It is possible that dextran-loaded lysosomes, the source of lysosomal membranes, include a small portion of dextran-loaded early and late endosomes, which may explain for the more association of rab 5 with lysosomal membranes.

The roles of trimeric GTP-binding proteins in the membrane fusion of lysosomes, the assembly of the Golgi apparatus, and the autophagic protein degradation of certain cell lines have recently drawn attention (28, 30, 31, 45). To accomplish these

![Fig. 3. Immunoblot analysis on BHMT and its partially degraded fragments, p35 and p32.](image)

**TABLE II**

| Spot | Name | Amino-terminal amino acid sequence |
|------|------|----------------------------------|
| o    | p32  | KISGGKVNEAAKDIARQVADIAVRQVADEGD |
| q    | p35  | YVAEIKISGGKVNEAAKD             |
| r    | p32  | KISGGKVNEAAKDIARQVADIAVRQVADEGD |
| u    | p44  | APIAGKAKRGILERLNGAEGVIGDDGFGVFALERKGYVAKAGPTPEAVE *

* Only the amino-terminal 10 residues could be determined for spot u separated by two-dimensional gel electrophoresis. The following 30 residues were determined for p44 immunoprecipitated using an anti-p44-peptide antibody (α-p44–10R).

**Fig. 4.** Nucleotide sequences of RT-PCR products and their deduced amino acid sequences. A, the nucleotide sequence of a cDNA clone obtained by RT-PCR followed by nested PCR is numbered from 1 to 232 above the nucleotide sequence. The deduced amino acid sequence is shown under the nucleotide sequence in single-letter code. Amino acids in bold letters indicate identity to the amino acid sequences determined at the protein level. Underlined nucleotide sequences indicate the synthetic primers used in nested PCR. Undetermined nucleotides and amino acids are represented by X. B, nucleotide and deduced amino acid sequences of a cDNA obtained by 5'3'-RACE are shown. The small lettered nucleotide sequence with negative numbers is the 5' noncoding sequence. Nucleotides in the coding region are shown in large letters and with positive numbers. Amino acid sequences identical to those determined at the protein level are in bold letters. The underlined nucleotide sequence indicates the Up-2 primer sequence. Nucleotides and amino acids indicated as X are undetermined.
functions, it is necessary for the GTP-binding proteins to become associated with target membranes. We confirmed that G\textsubscript{12α}, G\textsubscript{15α}, and G\textsubscript{α} are associated with both autolysosomal and lysosomal membranes. Although the exact functions of these molecules must be clarified by further investigations, it is of potential interest that G\textsubscript{α} is more abundant in the autolysosomal membranes.

Three polypeptides associated with autolysosomal membranes have been newly identified by RT-PCR as BHMT and its counterpart membranes (Fig. 5), which strongly suggests that the cleavage of p44 to yield p35 occurs in the cytosol. It has been reported previously (46) that the partial degradation of BHMT to produce low molecular mass forms occurs during purification and that this degradation can be inhibited by dimethylglycine and homocysteine, the product and substrate, respectively, of BHMT. In accordance with this observation, we performed immunoblotting to confirm that the addition of dimethylglycine and homocysteine (each 2 mM) to the homogenization buffer markedly reduces the p35 level in liver homogenates (data not shown). Furthermore, we could hardly detect p35 in primary cultured hepatocytes even under nutrient starvation conditions. These data indicate that the cleavage of p44 to produce p35 is an essential event, irrelevant to intra-autolysosomal proteolysis. It is likely that the homogenization of the liver causes the partial destabilization of BHMT in the absence of substrates or products, which enhances the susceptibility of the enzyme to cytosolic protease(s).

It should be noted that p32 is the first example of a limit-

Fig. 5. Location of p44, p35, and p32 in autolysosomes as revealed by immunoblot analysis using specific antibodies. A, autolysosomal protein (25 μg) was electrophoresed in 10% SDS-polyacrylamide gels and the separated proteins were electrophoretically transferred onto a Millipore membrane filter (GV, 0.22-μm pore size). The membrane strip was incubated with either an α-p44–10R (lane 1), α-p35–5R (lane 2), α-p32–5R (lane 3), or α-p32–10R (lane 4) antibody, followed by the hors eradish peroxidase conjugate of anti-rabbit IgG. The positions of molecular mass markers are shown on the left, while the positions of p44, p35, and p32 are shown on the right. B, autolysosomal membranes (lanes 1, 3, and 5) and the sediment fraction (lanes 2, 4, and 6), each containing 60 μg of protein, were electrophoresed in 10% SDS-polyacrylamide gels, and the separated proteins were electrophoretically transferred onto a Millipore membrane filter (GV, 0.22-μm pore size). The membrane strip was probed by immunoblotting with α-p44–10R (lanes 1 and 2), α-p35–5R (lanes 3 and 4), or α-p32–5R (lanes 5 and 6) antibodies. C, freshly prepared autolysosomes were incubated at 0 °C in medium (250 μl) containing 5 mM Tris (pH 7.5), 0.3 mM sucrose, and Pronase (0.4 mg/ml) in the presence (lanes 2, 6, and 9) or absence (lanes 2, 5, and 8) of 0.2% Triton X-100. As a control (lanes 1, 4, and 7), autolysosomes were incubated in medium without added Pronase or Triton X-100. The reaction was terminated by adding an equal volume of ice cold 10% trichloroacetic acid. The mixture was then centrifuged at 5,000 × g, and the pellets were solubilized and electrophoresed as described under “Experimental Procedures.” The separated proteins were electrophoretically transferred onto a Millipore membrane filter (GV, 0.22-μm pore size). The membrane strip was probed by immunoblotting using α-p44–10R (lanes 1–3), α-p35–5R (lanes 4–6), or α-p32–5R (lanes 7–9) antibodies.

quersted as a substrate for autophagy in autophagosomes and that p32 accumulates as a degradation product of p44 in the presence of leupeptin/E64c. In contrast, p35 is associated with the outer surface of the autolysosomal membrane (Fig. 5), which strongly suggests that the cleavage of p44 to yield p35 occurs in the cytosol. It has been reported previously (46) that the partial degradation of BHMT to produce low molecular mass forms occurs during purification and that this degradation can be inhibited by dimethylglycine and homocysteine, the product and substrate, respectively, of the BHMT reaction. In accordance with this observation, we performed immunoblotting to confirm that the addition of dimethylglycine and homocysteine (each 2 mM) to the homogenization buffer markedly reduces the p35 level in liver homogenates (data not shown). Furthermore, we could hardly detect p35 in primary cultured hepatocytes even under nutrient starvation conditions. These data indicate that the cleavage of p44 to produce p35 is an essential event, irrelevant to intra-autolysosomal proteolysis. It is likely that the homogenization of the liver causes the partial destabilization of BHMT in the absence of substrates or products, which enhances the susceptibility of the enzyme to cytosolic protease(s). In view of the data showing that p35 is also partially degraded fragments. BHMT catalyzes a reaction essential for the catabolism of betaine and homocysteine in mammalian liver, thus potentially affecting the regulation of methionine metabolism. BHMT is a major cytosolic enzyme consisting of six identical subunits (39). One intact subunit corresponds to p44 in this study. The two fragments, p35 and p32, lack the amino-terminal 87 and 91 residues, respectively, of p44. Since all the analyses of autolysosomal BHMT have been done by SDS-PAGE and immunoblots, we do not know whether these intact and partially degraded subunits exist as disassembled monomers or in polymeric forms. The Pronase digestion experiment and a close examination of the distribution of p44, p35, and p32 in the autolysosomal subfractions (Fig. 5) clearly demonstrated that p44 and p32 are present in the lumen of autolysosomes, showing that p44 (BHMT) is se-

Fig. 6. E64d-induced accumulation of a BHMT fragment (p32) in cultured hepatocytes under starvation conditions as demonstrated by immunoblots. A, hepatocytes cultured at 37 °C in 6-cm dishes with Williams E/10% FCS were washed twice and incubated with 5 ml of KRB buffer containing 0.1% dimethyl sulfoxide (control, lanes 1–5) and 10 μg/ml E64d plus 10 μg/ml pepstatin (lanes 6–10). At 2 h (lanes 1 and 6), 4 h (lanes 2 and 7), 8 h (lanes 3 and 8), 12 h (lanes 4 and 9), and 24 h (lanes 5 and 10) after the transition, cells were harvested and homogenized by sonication for 10 s in 0.5 ml of ice-cold 20 mM NaH\textsubscript{2}PO\textsubscript{4} (pH 7.5) containing 0.15 M NaCl. The homogenates were solubilized in SDS-PAGE sample buffer and electrophoresed in 10% SDS-polyacrylamide gels. The separated proteins were electrophoretically transferred onto a Millipore membrane filter (GV, 0.22-μm pore size). The membrane filter was incubated with either α-p32–10R (upper panel) or α-p32–5R (lower panel) antibody followed by the horseradish peroxidase conjugate of anti-rabbit IgG. B, hepatocytes cultured in 6-cm dishes with Williams E/10% FCS were washed with KRB buffer and incubated at 37 °C for 4 h with Williams E/10% FCS (lanes 4–6) or KRB buffer (lanes 1–3 and 7–9) containing the following reagents: 0.2% dimethyl sulfoxide (control, lanes 1 and 4); 10 μg/ml E64d (lane 2); 10 μg/ml E64d and 10 μg/ml pepstatin (lanes 3 and 5); 10 μg/ml E64d, 10 μg/ml pepstatin, and 0.2 μM rapamycin (lane 6); 10 μg/ml E64d, 10 μg/ml pepstatin, and 0.1 μM wortmannin (lane 7); 10 μg/ml E64d, 10 μg/ml pepstatin, 10 μM 3-methyladenine (lane 8); 10 μg/ml E64d, 10 μg/ml pepstatin, and 0.1 μM baflomycin (lane 9). After incubation, the hepatocytes were harvested, homogenized by sonication, solubilized, and electrophoresed in 10% SDS-polyacrylamide gels. The separated proteins were electrophoretically transferred onto a Millipore membrane filter (GV, 0.22-μm pore size). The membrane filter was incubated with either α-p32–10R (upper panel) or α-p32–5R (lower panel) antibody, followed by the horseradish peroxidase conjugate of anti-rabbit IgG.
digested intermediate of a sequestered protein found in autolysosomes. Inhibition of cysteine proteinases by leupeptin elicits the accumulation of autolysosomes holding various sequestered proteins, the substrates of autophagy, in their lumen, and these substrates can be recovered in the autolysosomal sediment fraction (23). As far as we have examined, none of these sequestered substrates have been found as limit-digested fragments. In other words, the sequestered proteins have the same mobilities in SDS-PAGE as their cytoplasmic counterparts. It is therefore difficult to use sequestered autophagy substrates as autolysosomal markers unless the autolysosomes can be clearly separated from other cytoplasmic components. However, the fact that p32 accumulation is strictly dependent on leupeptin/E64c indicates that its accumulation also parallels the accumulation of autolysosomes. As p32 can be easily and clearly distinguished from its cytoplasmic precursor (p44) in immunoblots, p32 in the cell homogenate can be used as a compelling autolysosomal marker. Immunoblot analysis using α-p32–10R and α-p32–5R confirms this idea and allowed us to develop a new, simple biochemical assay for starvation-induced autophagy as demonstrated by experiments performed with cultured hepatocytes (Fig. 6). When hepatocytes are incubated with KRB buffer to induce autophagy, p32 begins to accumulate, increases for 4 h after starvation, and reaches a plateau. The accumulation of p32 is totally dependent on E64d. Rapamycin, which enhances autophagy by inhibiting S6 kinase in both mammalian and yeast cells (37, 38), induces the accumulation of p32, because lysosomes have a common target of autolysosome formation or autolysosomal proteolysis when sequestered substrates have been found as limit-digested fragments. Inhibition of cysteine proteinases by leupeptin elicits the accumulation of autolysosomes holding various sequestered substrates. Inhibition of cysteine proteinases by leupeptin elicits the accumulation of autolysosomes holding various sequestered substrates.

REFERENCES

1. Schworer, C. M., Schiffer, K. A., and Mortimore, G. E. (1981) J. Biol. Chem. 256, 7652–7658
2. Seglen, P. O., Gordon, P. B., and Poli, A. (1980) Biochim. Biophys. Acta 630, 103–118
3. Seglen, P. O., and Bohey, P. B. (1992) Experience (Basel) 48, 158–172
4. Dunn, W. A. (1994) Trends Cell Biol. 4, 139–143
5. Dunn, W. A. (1990) J. Cell Biol. 110, 1923–1933
6. Yamamoto, A., Masaki, R., and Tashiro, Y. (1990) J. Histochem. Cytogenet. 38, 573–580
7. Seglen, P. O. (1987) in Lysosomes: Their Role in Protein Breakdown (Glaumann, H., and Ballard, F. J., eds) pp. 371–414, Academic Press Inc., London
8. Baba, M., Takeshige, K., Baba, N., and Ohsumi, Y. (1994) J. Cell Biol. 124, 101–118
9. Liu, W., Geuze, H. J., and Slot, J. W. (1996) J. Histochem. Cell Biol. 106, 51–58
10. Furuno, K., Ishikawa, T., and Kato, K. (1982) J. Biochem. (Tokyo) 91, 1485–1494
11. Furuno, K., Ishikawa, T., and Kato, K. (1982) J. Biochem. (Tokyo) 91, 1943–1950
12. Kominami, E., Hashida, S., Khairallah, E. A., and Katunuma, N. (1983) J. Biol. Chem. 258, 6093–6100
13. Ueno, T., Muno, D., and Kominami, E. (1991) J. Biol. Chem. 266, 18995–18999
14. Tanaka, K., Sato, M., Tomita, Y., and Ichihara, A. (1978) J. Biochem. (Tokyo) 84, 937–946
15. Laemmli, U. K. (1970) Nature 227, 680–685
16. Towbin, H., Staehelin, T., and Gordon, J. (1982) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
17. Kobayashi, R., and Tashima, Y. (1989) Anal. Biochem. 183, 9–12
18. Lusia, A. J., Tomono, S., and Paigen, K. (1976) J. Biol. Chem. 251, 7753–7760
19. Liu, F. T., Zinnecker, M., Hamaoka, T., and Katz, D. H. (1979) Biochemistry 18, 690–697
20. Chavrier, P., Parton, R. G., Hauri, H. P., Simons, K., and Zerial, M. (1990) Cell 62, 317–329
21. Muno, D., Ishidoh, K., Ueno, T., and Kominami, E. (1993) Arch. Biochem. Biophys. 306, 103–110
22. White, S., Miller, K., Hepkins, C., and Trowbridge, I. S. (1992) Biochim. Biophys. Acta 1136, 28–34
23. Furuno, K., Miyamoto, K., Ishikawa, T., and Kato, K. (1984) J. Biochem. (Tokyo) 95, 671–678
24. O’Farrell, P. Z., Goodman, H. M., and O’Farrell, P. H. (1977) Cell 12, 1133–1141
25. Huber, I. A., Pimplikar, S., Parton, R. G., Vitra, H., Zerial, M., and Simons, K. (1993) J. Cell Biol. 123, 35–45
26. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) Biochemistry 18, 5294–5299
27. Novick, P., and Brennwald, P. (1993) Cell 75, 597–601
28. Schwanninger, R., Plutner, R., Becková, G. M., and Balch, W. E. (1992) J. Cell Biol. 119, 1077–1096
29. Ward, D. M., Leslie, J. D., and Kaplan, J. (1997) J. Cell Biol. 138, 665–673
30. Jamora, C., Takizawa, P. A., Azarrou, R. F., Denesvre, C., Faulkner, D. J., and Malhotra, V. (1997) Cell 91, 617–626
31. Beron, W., Colombo, M. I., Mayorga, L. S., and Stahl, P. (1995) Arch. Biochem. Biophys. 317, 337–342
32. Maier, O., Rhenius, E., and Westermarck, P. (1995) Biochemical Biophys. Res. Commun. 205, 135–143
33. Garrow, T. A. (1996) J. Biol. Chem. 271, 22831–22838
34. Seglen, P. O., and Gordon, P. B. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 1389–1399
35. Blommaart, E. F. C., Krause, U., Seifert, J. P. M., Vreeling-Sindelarova, H., and Meijer, A. J. (1997) Eur. J. Biochem. 243, 240–246
36. Yamamoto, A., Tagawa, Y., Yoshimori, T., Moriyama, Y., Masaki, R., and Tashiro, Y. (1996) Cell Struct. Funct. 21, 33–42
37. Blommaart, E. F. C., Luiken, J. J. F. P., Blommaart, P. J. E., van Woerkom, G. M., and Meijer, A. J. (1995) J. Biol. Chem. 270, 2520–2526
38. Noda, T., and Ohsumi, Y. (1998) J. Biol. Chem. 273, 3963–3966
39. Masaki, R., Yamamoto, A., and Tashiro, Y. (1987) J. Cell Biol. 104, 1207–1215
40. Yamamoto, A., Masaki, R., Fukui, Y., and Tashiro, Y. (1990) J. Histochem. Cytogenet. 38, 1571–1575
41. Gordon, P. B., and Seglen, P. O. (1988) Biochemical Biophys. Res. Commun. 151, 40–47
42. Dunn, W. A. (1990) J. Cell Biol. 110, 1923–1945
43. Berg, T. O., Fengsrud, M., Stromhaug, P. E., Berg, T., and Seglen, P. O. (1998) J. Biol. Chem. 273, 21883–21892
44. Ullrich, O., Reinsch, S., Urbe, S., Zerial, M., and Parton, R. G. (1996) J. Cell Biol. 133, 913–924
45. Ogier-Denis, E., Covain, A., Mareset, J., Houri, J. J., Bavy, C., De Stefani, D., Ishidoro, C., Laburthe, M., and Codogno, P. (1995) J. Biol. Chem. 270, 15–16
46. Shibata, W., Wells, M. S., Mangum, J. H., and Awad, W. M., Jr. (1987) Methods Enzymol. 143, 384–388