

A 60 kDa Plasma Membrane Protein Changes its Localization to Autophagosome and Autolysosome Membranes during Induction of Autophagy in Rat Hepatoma Cell Line, H-4-II-E Cells

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ABSTRACT. We previously reported the preparation and characterization of an antibody against membrane fraction of autolysosomes from rat liver (*J. Histochem. Cytochem.* 38, 1571–1581, 1990). Immunoblot analyses of total membrane fraction of a rat hepatoma cell line, H-4-II-E cells by this antibody suggested that H-4-II-E cells expressed several autolysosomal proteins, including a protein with apparent molecular weight of 60 kDa. It was suggested that this 60 kDa protein was a peripheral membrane protein, because it was eluted from the membrane by sodium carbonate treatment. We prepared an antibody against this 60 kDa protein by affinity purification method, and examined its behavior during induction of autophagy. Autophagy was induced by transferring the cells from Dulbecco's modified Eagle medium (DMEM) containing 12% fetal calf serum into Hanks' balance salt solution. In DMEM, the 60 kDa protein showed diffused immunofluorescence pattern, and immunoelectron microscopy suggested that this protein was located on the extracellular side of the plasma membrane. After inducing autophagy, the immunofluorescence configuration of the 60 kDa protein changed from the diffused pattern to a granulous one. Immunoelectron microscopy showed that the 60 kDa protein was localized on the luminal side of the limiting membrane of autolysosomes and endosomes. In the presence of bafilomycin A₁ which prevents fusion between autophagosomes and lysosomes, the 60 kDa protein was localized on the limiting membrane of the autophagosomes and endosomes. These results suggest that the 60 kDa protein is transported from the plasma membrane to the autophagosome membrane through the endosomes.

Key words: autophagy/autophagosomes/plasma membrane/60 kDa protein/rat hepatoma cells/H-4-II-E cells

The balance between the synthesis and degradation of protein is essential in the control of growth and metabolism of cells. Autophagy is

one of the major pathways of degradation of intracellular proteins (18, 24). This process is observed ubiquitously in eukaryotic cells from yeast (3) to mammals (for review see 5, 8, 24), and is controlled by the nutritional condition of the cells and hormonal substances. For instance, it is well known that autophagy is immediately induced in perfused liver (21) or in cultured hepatocytes (23) after deprivation of amino acids.

At the early step of autophagy, a double membrane structure called isolation membrane sequesters a portion of cytoplasm and its two ends fuse with each other to form an autophagosome. The newly formed autophagosome is thought to mature into a more advanced

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Abbreviations: DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HBSS, Hanks' balanced salt solution; IEF, isoelectric focusing; PAGE, polyacrylamide gel electrophoresis; PB, phosphate buffer; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TBS, Tris-HCl buffered saline; V-ATPase, vacuolar proton ATPase.

stage of autophagosomes (late autophagosomes or amphisomes) of acidic luminal pH, acquiring vacuolar proton ATPase (V-ATPase) and other necessary proteins by fusion with endosomes or prelysosomes (2, 8, 11). In the next step, the autophagosomes are supplied with acid hydrolytic enzymes, and converted into autolysosomes, in which degradation of their content proceeds (7, 8, 33).

Although many studies have reported on the mechanism of autophagy, much remains unknown. Recently Tukada and Ohsumi isolated 14 genes essential for autophagy of yeast (28), and brought new and great progress in this field (19). The machinery for the formation of autophagosomes, however, has not been fully elucidated. For instance, the origin of the isolation membrane has yet to be determined, and the endoplasmic reticulum (6), the post-Golgi membrane with its complex type N-and/or O-linked oligosaccharide chains (30), and unique membrane structure called a phagophore (9) have been argued as the origin of the isolation membrane.

For the purpose of identification of protein components of autophagosome and autolysosome membranes, we previously prepared antibody against membrane fraction of rat liver autolysosomes, and showed that some proteins were specifically detected in autolysosome membranes, while some others were present both in autolysosome and in lysosome membranes (31).

Recently we developed a cultured cell system for studying autophagy using rat hepatoma cell line, H-4-II-E cells (32). H-4-II-E cells scarcely show autophagic activity in the presence of serum and amino acids, probably because these cells have high insulin sensitivity (12, 14). When cells were transferred into Hanks' balanced salt solution (HBSS) containing neither amino acids nor fetal calf serum (FCS), autophagy was induced, and autophagosomes and autolysosomes appeared in the cells within 1 hour (32). Using this cultured cell system, we showed that bafilomycin A₁, a potent inhibitor of V-ATPase, prevents fusion between autophagosomes and lysosomes, and causes accumulation of autophagosomes in the cells (32).

In this study, we analyzed the process of autophagy in H-4-II-E cells, using antibody against membrane fraction of autolysosomes from rat liver. We found that the H-4-II-E cells express a 60 kDa protein recognized by this antibody as a major band in immunoblot analysis. On examining the behavior of this protein during the induction of autophagy, we found that it was transferred from plasma membrane to the limiting membranes of autophagosomes and autolysosomes probably through the endosomes. These results suggest the possibility that some plasma membrane proteins participate in the formation or maturation of the autophagic system.

Materials and Methods

Materials

Preparation of rabbit polyclonal antibody against membrane fraction of the autolysosomes (autolysosome membrane) from rat liver (31), cathepsin B (15), and α -subunit of Na, K-ATPase (1) has been described previously. Mouse monoclonal antibody against rat transferrin receptor was purchased from Serotec Inc., Oxford, UK. Bafilomycin A₁ was kindly provided by Prof. K. Altendorf (Universität Osnabrück, Germany). Bafilomycin A₁ was dissolved in dimethyl sulfoxide (DMSO) and stored at -80°C .

Cell culture

A cell line, H-4-II-E-C3 cells (ATCC No. CRL-1600) derived from rat hepatoma H35 cells, was used throughout this study. H-4-II-E cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 12% FCS. To induce autophagy, cells were incubated in HBSS containing 40 mM Hepes-NaOH buffer pH 7.4. For bafilomycin A₁ treatment experiments, 1/100 volume of bafilomycin A₁ dissolved in DMSO was added to the medium.

Preparation of total membrane fraction of H-4-II-E cells

Cells were homogenized in 0.25 M sucrose buffer (0.25 M sucrose, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.2 mg/ml antipain, 0.2 mg/ml leupeptin, 100 units/ml aprotinin, 2 mM (p-amidinophenyl) methanesulfonyl fluoride hydrochloride (p-APMSF), 20 $\mu\text{g}/\text{ml}$ chymostatin, 20 $\mu\text{g}/\text{ml}$ pepstatin, 0.5 mg/ml soybean trypsin inhibitor) and loaded on 500 μl of 1 M sucrose buffer (1 M sucrose, 10 mM Tris-HCl (pH 7.5), 10 mM KCl, 0.5 mM MgCl_2), and centrifuged at $700\times g$ for 10 min at 4°C . The supernatant was centrifuged at $101,000\times g$ for 90 min at 4°C . The pellet (total membrane fraction) was collected and suspended in 1 M sucrose buffer. After protein concentration was determined by Bio-Rad protein assay kit (Bio-Rad lab. Hercules, CA) using bovine gamma globulin as a standard, the total membrane fraction was stored at -80°C .

Na_2CO_3 treatment

Four hundred μl of total membrane fraction of H-4-II-E cells (100 μg protein) was mixed into 400 μl of 200 mM Na_2CO_3 (pH 11.5), and incubated for 30 min at 4°C (10), followed by centrifugation of the suspension at $88,000\times g$ for 80 min at 4°C . The supernatant was precipitated with 10% TCA, washed twice with 90% ethanol and once with diethylether, and then dried.

Preparation of antibody against the 60 kDa Protein by affinity purification

Proteins in the total membrane fraction of H-4-II-E cells were separated with SDS-polyacrylamide gel electrophoresis (PAGE) (16), and transferred to GVHP sheet (Nihon Millipore Kogyo, Yonezawa, Japan). The part of the GVHP sheet corresponding to the 60 kDa in mobility was cut out. A piece of this sheet containing the 60 kDa protein was incubated with 5% bovine serum albumin (BSA) in 50 mM Tris-HCl-buffered saline pH 7.5 (TBS) overnight at 4°C, and then incubated with antibody against autolysosome membrane overnight at 4°C. The bound antibody was released from the sheet in 0.2 M glycine-HCl buffer (pH 3.3). The antibody was neutralized with 3 M Tris-HCl (pH 8.8) immediately, and stored at 4°C.

Immunoblot Analysis

Total membrane fraction of H-4-II-E cells was separated by SDS-PAGE under reducing condition. The electrophoresed proteins were transferred to a GVHP sheet and were incubated overnight at 4°C with 5% skim milk in TBS, and then overnight with the antisera against autolysosome membrane or affinity purified antibody against the 60 kDa protein dissolved in TBS containing 1% skim milk and 0.05% Tween 20. After washing in TBS containing 0.05% Tween 20, the sheet was incubated with goat IgG against rabbit IgG conjugated with horseradish peroxidase. After washing in TBS containing 0.05% Tween 20, the antigen-IgG complex was visualized with enhanced chemiluminescence (ECL) system (Amersham, Buckinghamshire, UK).

Two dimensional gel electrophoresis

For O'Farrel 2 dimensional gel electrophoresis (20), total membrane fraction of H-4-II-E cells (5 µg protein) was applied onto one-dimensional gel (immobilized pH gradient gel, Pharmacia, Uppsala, Sweden), and isoelectric focusing (IEF) was performed overnight (22,650 voltage × hours). After the gel was incubated in SDS sample buffer for 30 min, proteins were separated by SDS-PAGE.

Immunofluorescence microscopy

Indirect immunofluorescence microscopy was performed according to the procedure of Saga *et al.* (22) with slight modification. H-4-II-E cells were cultured on cover slips. The cells were fixed with 4% paraformaldehyde in PBS containing 5% sucrose, 1 mM CaCl₂ and 0.5 mM MgCl₂ (PBS(+)), and were permeabilized with 0.2% Triton-X in PBS(+) for 30 min. After washing with PBS(+), non-specific binding was blocked with 3% BSA, 0.1 M glycine, and 10% goat serum in PBS(+) for 30 min. The cells were incubated in the affinity-purified antibody against the 60 kDa protein, Na, K-ATPase, cathepsin B, or mouse monoclonal antibody against transfer-

rin receptor dissolved in PBS(+) containing 10% goat serum for 60 min. After rinsing in PBS(+), the cells were then reacted with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (or anti-mouse IgG in case of antibody against transferrin receptor) dissolved in PBS(+) containing 10% goat serum for 30 min. The coverslips were rinsed in PBS(+), and were observed under an Olympus fluorescence microscope (BH-2; Olympus Corp., Tokyo, Japan).

Immunoelectron microscopy

Immunogold labeling on the cryo-ultrathin sections was carried out according to the method of Tokuyasu (25, 26). H-4-II-E cells were collected by centrifugation, and the pellet was fixed for 15 min in 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.4 (PB), and incubated overnight in 2.3 M sucrose in PB containing 20% polyvinyl pyrrolidone, and then rapidly frozen in liquid nitro-

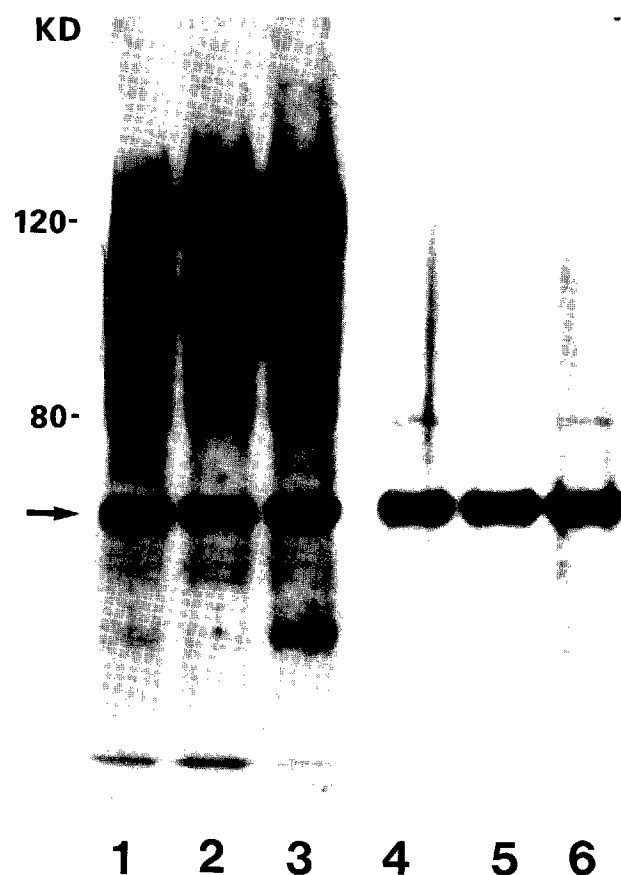


Fig. 1. Immunoblot analysis of total membrane fraction of H-4-II-E cells with antibody against autolysosome membrane (lane 1–3) or with affinity purified antibody against the 60 kDa protein (lane 4–6). Total membrane fraction was prepared from H-4-II-E cells cultured in DMEM containing FCS (lane 1, 4), in HBSS for 1 hour (lane 2, 5), and in HBSS containing 100 nM bafilomycin A₁ for 1 hour (lane 3, 6). The arrow shows 60 kDa protein.

gen, and cut with a Reichert Ultracut E with a cryoattachment (FC-4D) at -100°C to a thickness of 80 nm. The sections were picked up on formvar-carbon-coated nickel grids (150 mesh). The grids were washed in a drop of PBS containing 10 mM glycine, incubated for 5 min at 37°C in PBS containing 2% gelatin and 10 mM glycine, and then incubated for 30 min at 37°C with antibody against the 60 kDa protein. The grids were then washed six times in PBS containing 2% gelatin and 10 mM glycine, and reacted for 15 min at 37°C with gold particles conjugated with goat IgG against rabbit IgG (10 nm in diameter, Ultra Biosols, Liverpool, UK). After washing with 0.1 M sodium cacodylate buffer, pH 7.4, the sections were post-fixed in 2% glutaraldehyde for 10 min. After washing in distilled water, sections were stained and embedded in 1.5% uranyl acetate containing 1.5% polyvinylalcohol (26), and observed under a Hitachi H7000 electron microscope (Hitachi, Tokyo, Japan).

Results

Immunochemical analysis of H-4-II-E cells using antibody against autolysosome membrane

First we studied whether rat hepatoma cells express liver autolysosome membrane proteins or not. Figure 1 shows immunoblot analyses of the total membrane fraction of H-4-II-E cells by the antibody against rat liver autolysosome membrane. Several bands showing the presence of the autolysosome membrane proteins

appeared, including two major bands at 80–120 kDa and 60 kDa in apparent molecular weight (MW) in cells cultured in DMEM containing 12% FCS (Fig. 1, lane 1). When cells were transferred into HBSS containing neither amino acids nor FCS, autophagy was induced, and autophagosomes and autolysosomes appeared in the cells within 1 hour (32). Bafilomycin A_1 prevents fusion between autophagosomes and lysosomes, and causes accumulation of autophagosomes in the cells (32). The pattern of the immunoblot did not change after induction of autophagy by transferring the cells into HBSS and after incubation of the cells in HBSS containing 100 nM bafilomycin A_1 (Fig. 1, lanes 2 and 3). The broad 80–120 kDa band was separated into several bands when exposure time in ECL was shortened (data not shown). This result suggested that it consists of plural bands overlapping each other. These bands were considered to correspond to already-known lysosomal membrane glycoproteins including LGP 107 (13, 31). To characterize another major band of 60 kDa in MW, we affinity-purified antibody against the 60 kDa protein from the antibody against autolysosomal membrane. This affinity-purified antibody showed only 60 kDa band in immunoblot analysis of the total membrane fraction of H-4-II-E cells (Fig. 1, lane 4–6). The 60 kDa protein was not detected in the supernatant of total membrane fraction or in the culture medium (data not shown).

The amount of the 60 kDa protein did not change

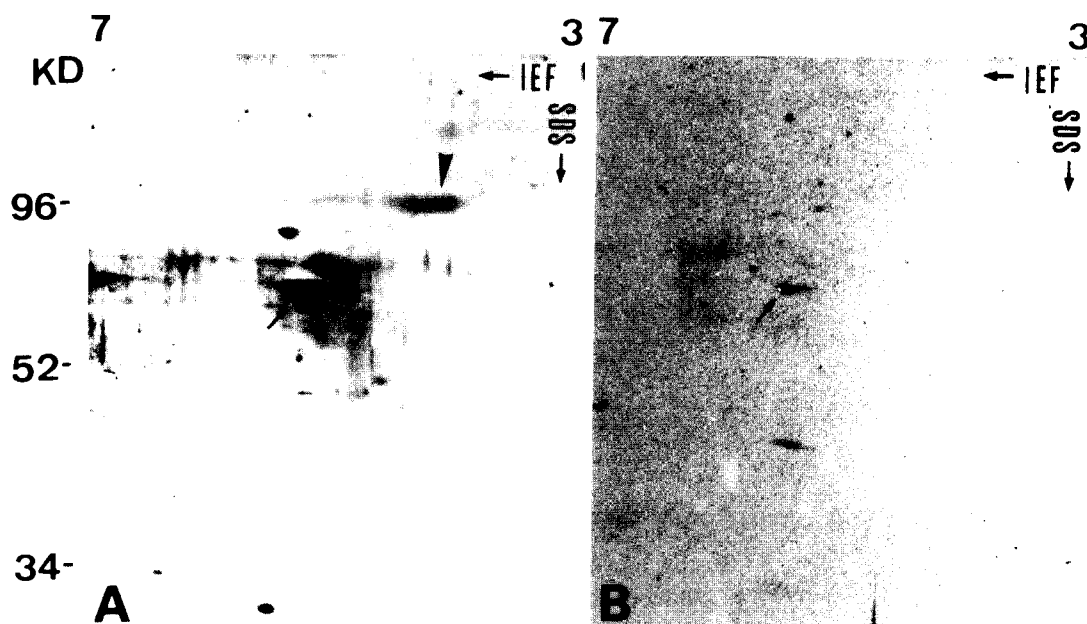


Fig. 2. Two dimensional immunoblot analysis. Total membrane fraction of H-4-II-E cells cultured in DMEM containing FCS was separated by two dimensional gel electrophoresis and reacted with antibody against autolysosome membrane (A) or with the affinity-purified antibody against the 60 kDa protein (B). Arrows show 60 kDa protein. The arrowhead indicates LGP 107.

during induction of autophagy by transferring the cells to HBSS from DMEM or after incubation of the cells in HBSS containing 100 nM bafilomycin A₁ (Fig. 1, lane 4–6). Pulse label experiment using ³⁵S methionine also showed that there was no change in the synthesis of the 60 kDa protein during induction of autophagy (data not shown).

Next we checked the isoelectric point of the 60 kDa protein using two-dimensional gel electrophoresis. Using antibody against autolysosome membrane, several spots including the 60 kDa protein and highly acidic protein with broad isoelectric point (4.2–5.6) appeared (Fig. 2A). Judging from its molecular weight and isoelectric point, the acidic protein may correspond to highly sialylated lysosomal glycoprotein, LGP 107 (13). By using antibody against the 60 kDa protein, one clear spot appeared at about 5.6 in isoelectric point (Fig. 2B). The results strongly suggested that this affinity-purified antibody specifically recognized a single membrane protein.

The 60 kDa protein is a peripheral membrane protein

Next, we examined whether this protein was tightly anchoring or loosely binding to the membrane using sodium carbonate treatment (Fig. 3). Total membrane fraction of the cells cultured in HBSS for 1 hour was treated with 100 mM sodium carbonate, and the resultant pellet and supernatant were analyzed by immunoblot with the antibody against autolysosome membrane and with the affinity purified antibody against the 60 kDa protein. As expected 80–120 kDa proteins were mainly recovered from the pellet (Fig. 3A). In contrast, the 60 kDa protein was eluted to soluble fraction showing that it is a peripheral membrane protein (Fig. 3B).

Immunofluorescence localization of the 60 kDa protein during induction of autophagy

Next we investigated the behavior of the 60 kDa protein during the induction of autophagy by immunofluorescence microscopy using the affinity purified antibody. H-4-II-E cells grow usually forming colonies consisting of 2–10 cells. To our surprise, the 60 kDa protein showed diffused localization pattern in the cells cultured in DMEM containing FCS (Fig. 4A). A similar diffuse pattern was observed when cells were not permeabilized before immunostaining (Fig. 4E). No remarkable differences in the strength of immunofluorescence were detected between permeabilized and non-permeabilized cells. These results suggested that the 60 kDa protein is a plasma membrane protein located on the outer surface of the cells.

The diffuse plasma membrane staining in DMEM changed into granular pattern after 15–30 min. of incu-

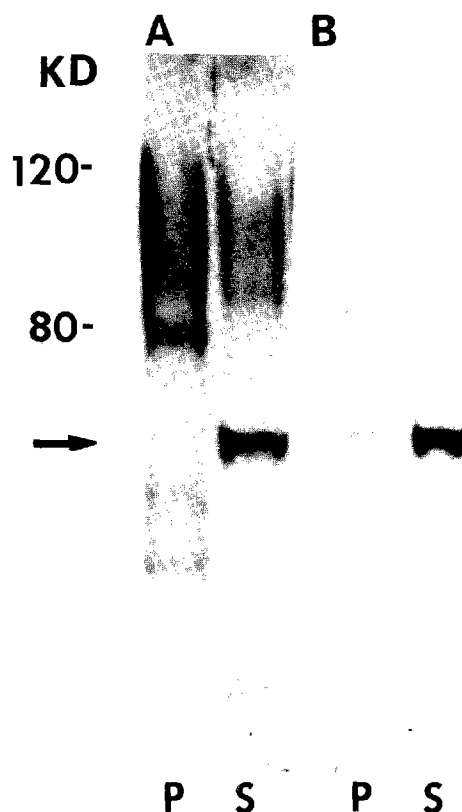


Fig. 3. Na₂CO₃ treatment of the 60 kDa protein. Total membrane fraction of the cells cultured in HBSS for 1 hour was treated with 100 mM sodium carbonate, and pellet (P) and supernatant (S) were analyzed by immunoblot with antibody against autolysosome membrane (Fig. 3A) or with the affinity purified antibody against the 60 kDa protein (Fig. 3B). The 60 kDa protein is exclusively detected in the soluble fraction. The arrow shows 60 kDa protein.

bation in HBSS (Fig. 4B, C). After 1 hour of incubation in HBSS, most of the cells showed granular staining (Fig. 4D). When cells were not permeabilized, surface staining of the 60 kDa protein decreased during induction of autophagy (Fig. 4E–H), suggesting that the 60 kDa protein moves into intracellular granules from the plasma membrane.

Figure 5 shows immunofluorescence labeling of the 60 kDa protein and a lysosomal enzyme, cathepsin B after 1 hour of incubation of the cells in HBSS or in HBSS containing 100 nM bafilomycin A₁. Addition of bafilomycin A₁ to HBSS did not inhibit appearance of the granular staining of the 60 kDa protein (Fig. 5C). Cathepsin B showed punctate staining pattern, which is characteristic of lysosomes, in cells cultured in DMEM containing FCS (Fig. 5D). When cells were transferred into HBSS, cathepsin B also showed punctate pattern, but the size of cathepsin B positive structures became larger than those in DMEM (Fig. 5E). The increase in size corresponds to the formation of autolysosomes by

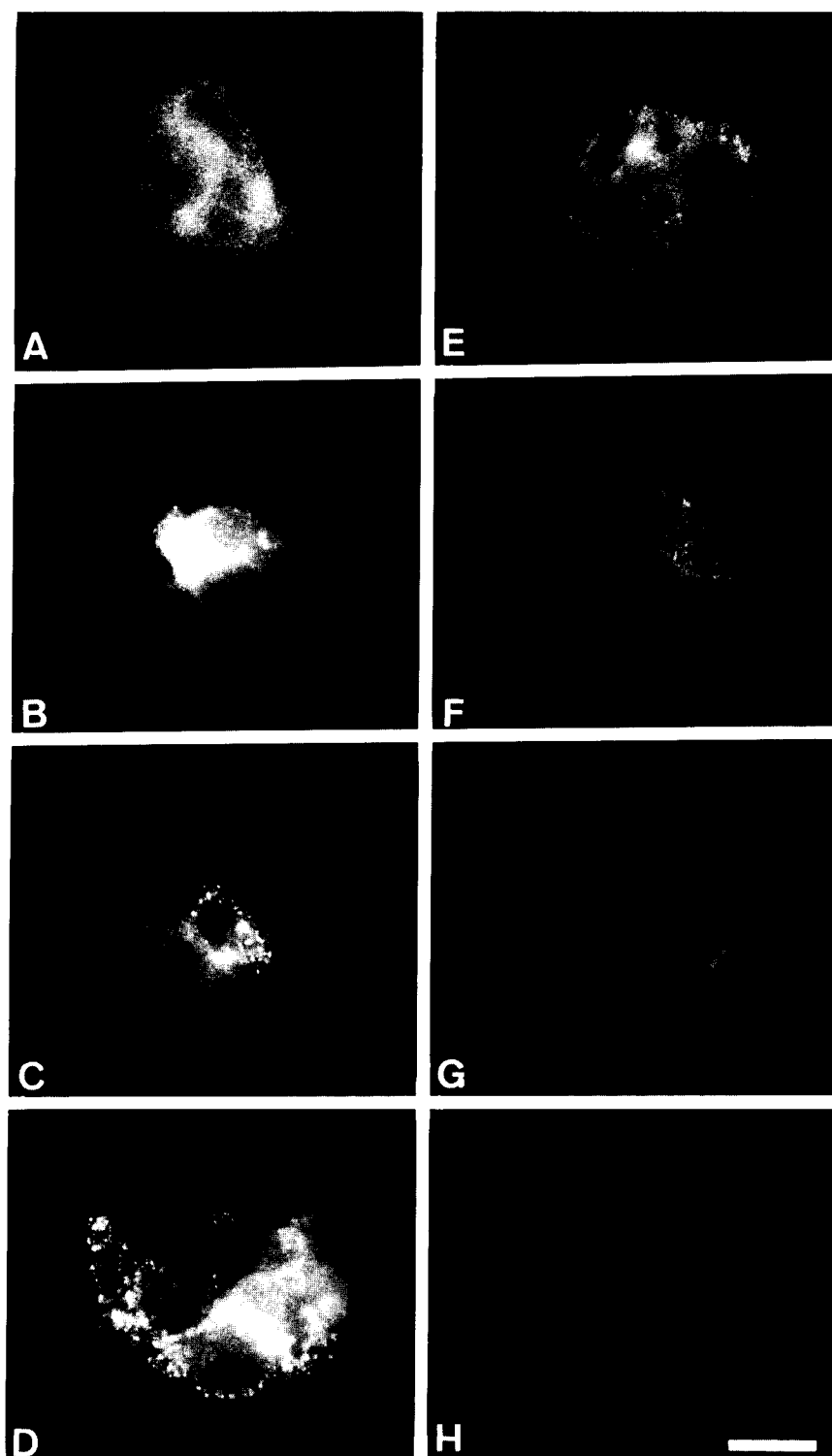


Fig. 4. Immunofluorescence localization of the 60 kDa protein during induction of autophagy. The 60 kDa protein was detected in H-4-II-E cells cultured in DMEM containing FCS (A, E), 15 min. (B, F), 30 min. (C, G), and 60 min. (D, H) after transfer of the cell into HBSS. Cells were immunostained with affinity-purified antibody against the 60 kDa protein after (A–D) or before (E–H) permeabilization with Triton X-100. Colonies consisting of several cells are shown. Bar, 20 μ m.

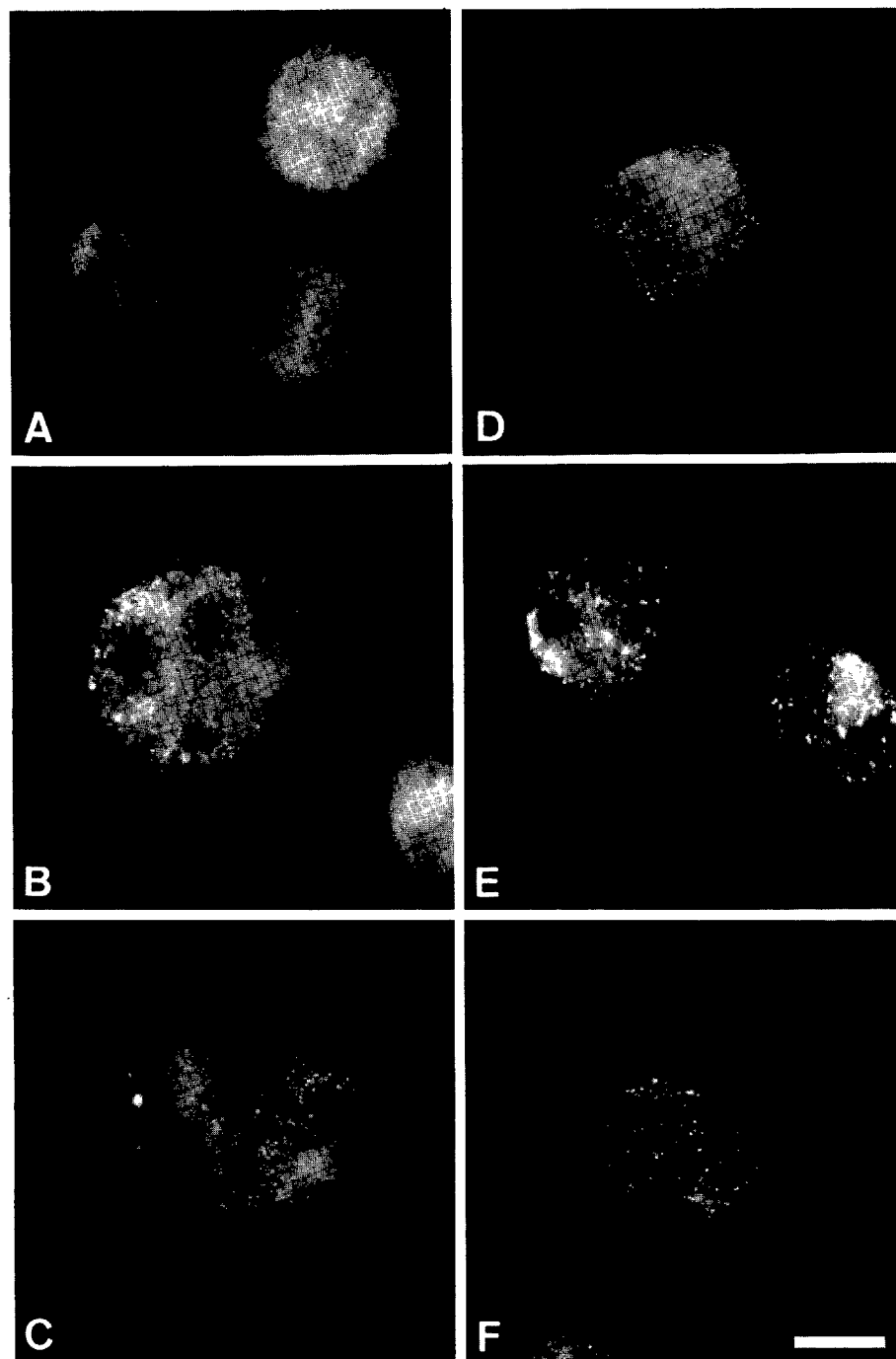


Fig. 5. Immunofluorescence localization of the 60 kDa protein and cathepsin B during induction of autophagy. The 60 kDa protein (A–C) and cathepsin B (D–F) were detected in H-4-II-E cells cultured in DMEM containing FCS (A, D), after 1 hour of incubation of the cells in HBSS (B, E) or in HBSS containing 100 nM bafilomycin A₁ (C, F) after permeabilization with Triton X-100. Colonies consisting of several cells are shown. Bar, 20 μ m.

fusion between autophagosomes and lysosomes. In the presence of bafilomycin A₁, when fusion between autophagosomes and lysosomes was inhibited, the size of

cathepsin B positive structures did not increase (Fig. 5F). In HBSS, the staining pattern of 60 kDa protein was very similar to that of cathepsin B, suggesting that

the 60 kDa protein was localized in autolysosomes during induction of autophagy.

Immunoelectron microscopic localization of the 60 kDa protein during induction of autophagy

We further examined the localization of the 60 kDa protein during induction of autophagy using immunoelectron microscopy. In the H-4-II-E cells cultured in DMEM containing FCS, gold particles showing the presence of the 60 kDa protein were mainly detected on the extracellular surface of the plasma membrane (Fig. 6). This result was consistent with the result obtained by immunofluorescence method, and strongly suggested that the 60 kDa protein is a plasma membrane protein located on the outer surface of the cells.

In the H-4-II-E cells cultured in HBSS for 1 hour, there appeared many autolysosomes which contained electron dense material in the process of degradation (32). The 60 kDa protein was localized on the luminal

surface of the limiting membrane of autolysosomes and of endosomes (Fig. 7A). On the other hand, labeling on the plasma membrane became very low after 1 hour incubation in HBSS (Fig. 7A). In the presence of bafilomycin A₁, autophagosomes which contained intact cytoplasm accumulated instead of autolysosomes (32). The 60 kDa protein was localized on the limiting membrane of the autophagosomes and of endosomes (Fig. 7B). The localization of the 60 kDa protein in endosomes suggested that this protein was transported from the plasma membrane to the autophagosomes and autolysosomes through the endosomes.

Behavior of other plasma membrane proteins on autophagy

We examined whether change of location from the plasma membrane to autolysosomes occurs in other plasma membrane proteins using immunofluorescence microscopy. Figure 8 shows the localization of the α -

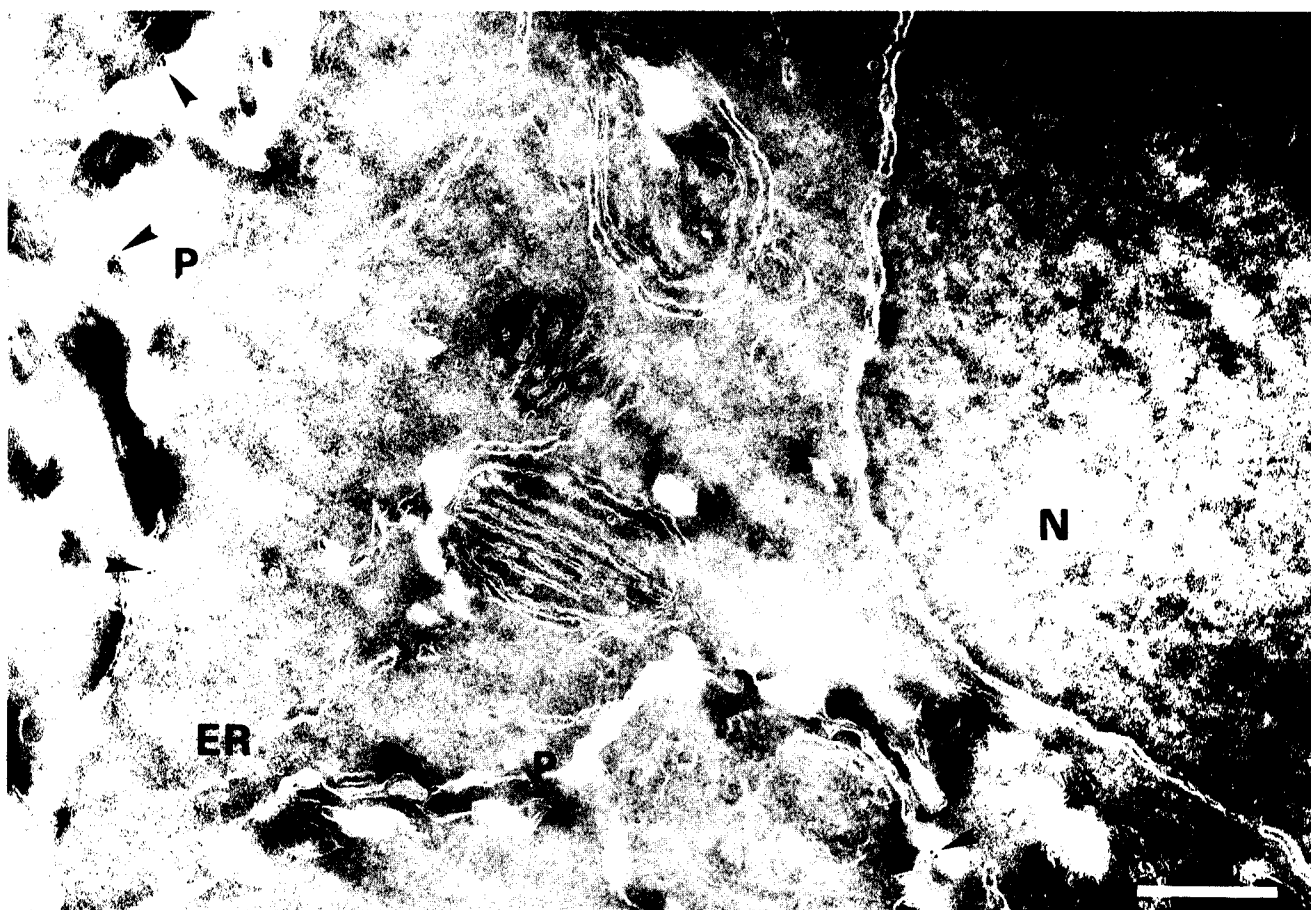


Fig. 6. Immunoelectron microscopic localization of the 60 kDa protein in H-4-II-E cells cultured in DMEM containing FCS. Cryoultrathin sections were stained using an immunogold technique. Gold particles (arrowheads) showing the presence of the 60 kDa protein were mainly located on the extracellular surface of the plasma membrane (P). ER; endoplasmic reticulum, N; nucleus, Bar, 500 nm.

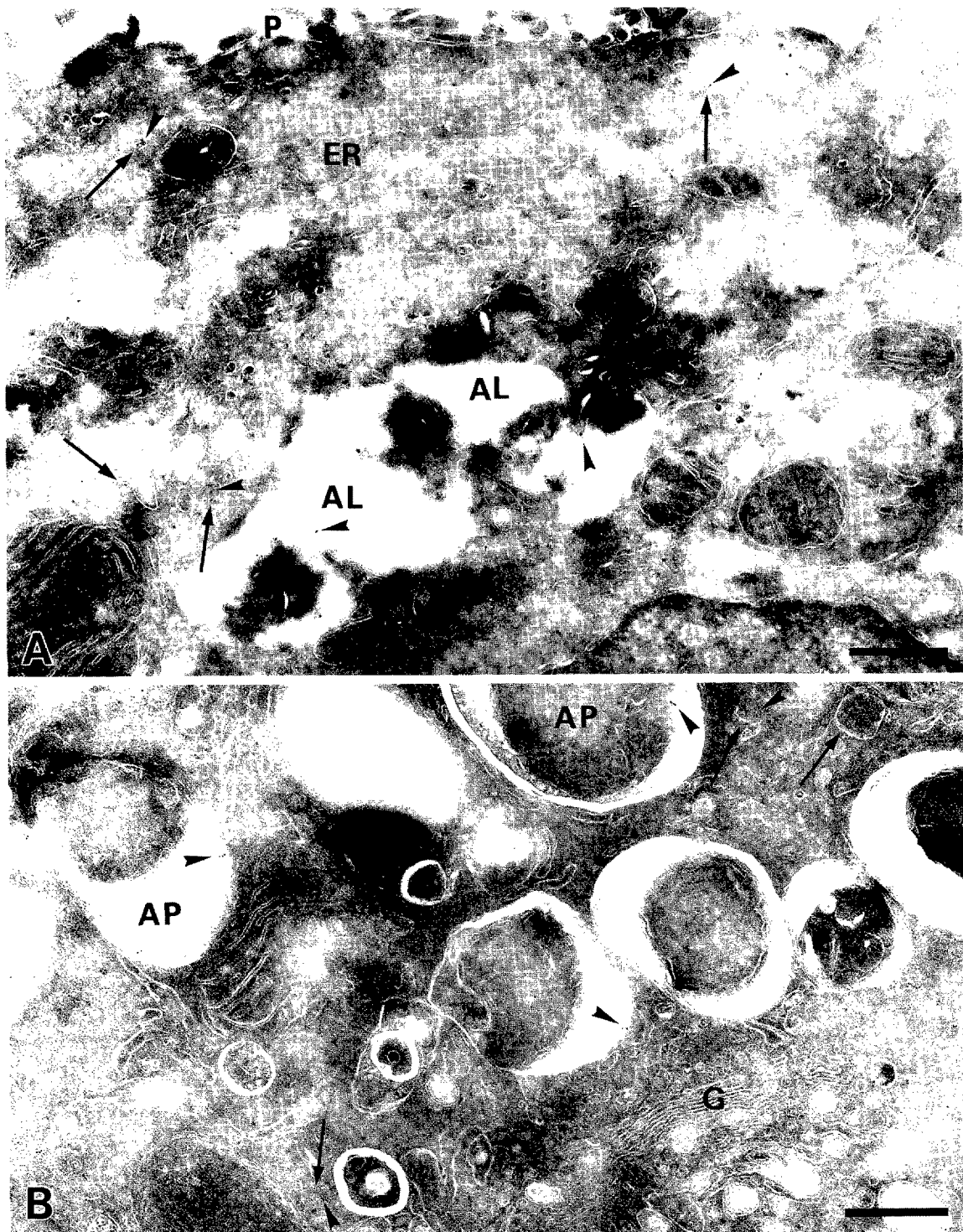


Fig. 7. Immunoelectron microscopic localization of the 60 kDa protein in H-4-II-E cells after induction of autophagy. A: Cells were cultured in HBSS for 1 hour. The 60 kDa protein is localized on the luminal surface of the limiting membrane of autolysosomes (AL) and of endosomes (arrows). The labeling on the plasma membrane (P) is very low. B: Cells were cultured in HBSS containing 100 nM bafilomycin A₁ for 1 hour. The 60 kDa protein is localized on the limiting membrane of the autophagosomes (AP) and of endosomes (arrows). Arrowheads indicate gold particles. ER; endoplasmic reticulum, G; Golgi apparatus, Bars, 500 nm.

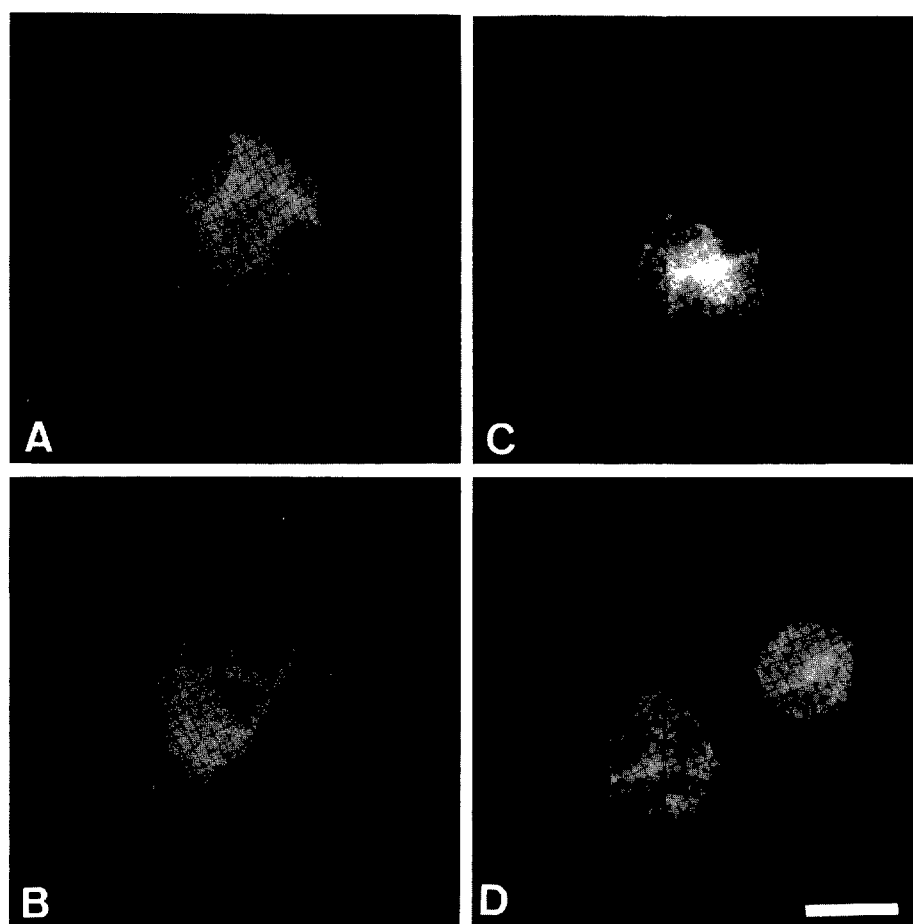


Fig. 8. Immunofluorescence localization of Na, K-ATPase and transferrin receptor during induction of autophagy. Na, K-ATPase (A, B) and transferrin receptor (C, D) were detected in H-4-II-E cells cultured in DMEM containing FCS (A, C), and after 1 hour of incubation of the cells in HBSS (B, D) after permeabilization with Triton X-100. Colonies consisting of several cells are shown. Bar, 20 μ m.

subunits of Na, K-ATPase and transferrin receptor before and after induction of autophagy. In DMEM containing FCS, Na, K-ATPase was localized on the plasma membrane, and transferrin receptor was detected in endosome-like structures as well as the plasma membrane. No remarkable differences were observed in Na, K-ATPase (Fig. 8A, B) and transferrin receptor (Fig. 8C, D) during induction of autophagy.

Discussion

In this paper, we have reported the interesting behavior of a plasma membrane protein during autophagy. A 60 kDa protein, which is usually localized on the plasma membrane, is transferred into the autophagosomes and autolysosomes during induction of autophagy in H-4-II-E cells.

The 60 kDa protein is one of the proteins recognized by antibody against autolysosomal membrane. The lo-

calization of this protein on the plasma membrane was something unexpected. However, this is not so peculiar because autolysosomal membrane antigens have also been detected on the plasma membrane, Golgi apparatus, and lysosomes as well as in autolysosomes and autophagosomes in rat hepatocytes (31). Immunofluorescence and immunoelectron microscopy suggested that the 60 kDa protein is a peripheral membrane protein located on the extracellular side of the plasma membrane, and that after induction of autophagy, it is localized on the luminal surface of the limiting membrane of the autolysosomes. The topology of the 60 kDa protein suggests that this protein is transported from the plasma membrane to the autophagosomes via vesicular traffic such as endocytosis. This possibility is strongly supported by the immunoelectron microscopic observation that the 60 kDa protein appeared in the endosomes after the induction of autophagy. The change of localization was specific for the 60 kDa pro-

tein, while other membrane proteins, Na, K-ATPase and transferrin receptor did not show any change in the distribution in H-4-II-E cells after induction of autophagy.

The convergence of autophagic and endocytic pathways has been reported by several investigators (7, 11, 17, 27). Therefore it is very possible that newly formed autophagosomes acquire the 60 kDa proteins by fusion with endosomes. Bafilomycin A₁, a macrolide antibiotics isolated from *Streptomyces* sp. (29), is known to be a specific inhibitor of V-ATPase and effective at nM concentrations in vivo (34). We previously reported that this drug prevents fusion between autophagosomes and lysosomes in H-4-II-E cells, and suggested that the acidity of the autophagosomes is important for the fusion between them (32). The 60 kDa protein moved into the autophagosomes in the presence of bafilomycin A₁. This result suggests that this drug did not prevent fusion between autophagosomes and endosomes.

Although the function of the 60 kDa protein is unknown at present, it is plausible that it plays a role in the formation or maturation of the autophagosomes. Another possibility that 60 kDa protein might have been transported into autolysosomes for degradation seems unlikely, because the amount of the 60 kDa protein in immunoblot analysis did not decrease after 1 hour of incubation in HBSS while a considerable part of the 60 kDa protein was transported into the autolysosomes by then.

Recently, Baricault *et al.* reported that forskolin treatment causes rapid sequestration of plasma membrane proteins, dipeptidyl peptidase IV, 525 antigen, and sucrase-isomaltase from cell surface into intracellular autophagic vacuole-like compartment in Caco-2 cells (4). Their study also suggests important roles of plasma membrane proteins in the autophagic process, although in the case of forskolin treatment, the sequestration of plasma membrane proteins was thought to result from changes in exocytic pathway rather than from the increase of endocytosis of these proteins.

In conclusion, we found a 60 kDa plasma membrane protein which moves into autophagosomes during induction of autophagy. Until now, reliable markers for autophagosomes have not been reported yet. The 60 kDa protein will become a useful marker for autophagy as well as a useful tool for elucidating the mechanism of autophagy.

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