Amphisomes, the autophagic vacuoles (AVs) formed upon fusion between autophagosomes and endosomes, have so far only been characterized in indirect, functional terms. To enable a physical distinction between autophagosomes and amphisomes, the latter were selectively density-shifted in sucrose gradients following fusion with AOM-gold-loaded endosomes (endosomes made dense by asialoorosomucoid-conjugated gold particles, endocytosed by isolated rat hepatocytes prior to subcellular fractionation). Whereas amphisomes, by this criterion, accounted for only a minor fraction of the AVs in control hepatocytes, treatment of the cells with leupeptin (an inhibitor of lysosomal protein degradation) caused an accumulation of amphisomes to about one-half of the AV population. A quantitative electron microscopic study confirmed that leupeptin induced a severalfold increase in the number of hepatocytic amphisomes (recognized by their gold particle contents; otherwise, their ultrastructure was quite similar to autophagosomes). Leupeptin caused, furthermore, a selective retention of endocytosed AOM-gold in the amphisomes at the expense of the lysosomes, consistent with an inhibition of amphisome-lysosome fusion. The electron micrographs suggested that autophagosomes could undergo multiple independent fusions, with multivesicular (late) endosomes to form amphisomes and with small lysosomes to form large autolysosomes. A biochemical comparison between autophagosomes and amphisomes, purified by a novel procedure, showed that the amphisomes were enriched in early endosome markers (the asialoglycoprotein receptor and the early endosome-associated protein 1) as well as in a late endosome marker (the cation-independent mannose 6-phosphate receptor). Amphisomes would thus seem to be capable of receiving inputs both from early and late endosomes.

Autophagy is a mechanism by which cells sequester and degrade parts of their own cytoplasm, including organelles (for a review, see Ref. 1). Autophagy is basically a nonselective, bulk process; a number of cytosolic enzymes with widely different half-lives have been shown to be autophagically sequestered at identical rates (2). However, a selective organelle autophagy can apparently take place during the regression of hypertrophied organelles like the peroxisomes (3) and by the smooth endoplasmic reticulum (4). In the first recognizable autophagic step, single or multiple membrane cisternae with a distinct osmiophilic morphology (5–7), called phagophores (8, 9), wrap up a region of cytoplasm into a closed vacuolar organelle, an autophagosome. The phagophores, which form the autophagosome wall, may ultimately be derived from the endoplasmic reticulum (10–13), perhaps representing highly modified endoplasmic reticulum cisternae that have acquired unique biochemical and structural properties (5, 13–16).

Biochemical studies have indicated that autophagosomes can fuse with endosomes to form prelysosomal autophagic/ endocytic vacuoles called amphisomes (17–19). (Autophagosomes, amphisomes, and lysosomes engaged in autophagy are often referred to collectively as autophagic vacuoles (AVs)1). The amphisomes are apparently acidic (20), probably due to the activity of a proton pump contributed by the endosomal fusion partner. Morphologically, hepatocytic amphisomes can be recognized as “intermediate” autophagic vacuoles (AVid) (i.e. autophagosome-like vacuoles containing endocytic markers like endocytosed gold or albumin-gold; acidic, yet lacking lysosomal enzymes or lysosomal membrane proteins like the glycoprotein Lgp120 (9, 12, 21)). Amphisome formation has also been demonstrated in nonhepatic cells and tissues (22, 23).

There is good evidence that some of the autophagosomes may fuse directly with lysosomes (6, 12, 19, 21, 24, 25). It has been suggested that autophagic delivery to the lysosomes may occur exclusively by autophagosome-lysosome fusion, i.e. without amphisome formation (24, 25), but a recent morphometric study concluded that hepatocytic endosomes were 5–6 times more likely to enter the amphisome pathway than to fuse directly with lysosomes (21).

Differences in experimental approach, organelle markers, and organelle definitions make it difficult to resolve discrepant conclusions regarding the relative roles of the amphisomal and the direct autophagosomal pathway to the lysosome. Active lysosomes can be distinguished from the double- or multiple-membrane-enclosed autophagosomes by having only a single delimiting membrane, but amphisomes have not yet been unequivocally characterized in morphological terms. We therefore initiated the present study, using colloidal gold particles conjugated to asialoorosomucoid (AOM) both as an ultrastructural marker and as a density perturbant. AOM-gold is taken up by receptor-mediated endocytosis in isolated rat hepatocytes, and by means of different gold particle sizes and different loading times, a differential staining of lysosomes and prelysosomal
endocytic vacuoles (which would include the amphisomes) can be obtained (26, 27). In addition, the high density of the gold particles would be expected to change the behavior of gold-laden endocytic vacuoles in density gradients, as has been shown in other cell types (28). By this approach, we have been able to identify the amphisomes both as a structural entities in sucrose density gradients and as distinct vacuoles in electron micrographs.

**EXPERIMENTAL PROCEDURES**

*Biochemicals—Tyramine-cellobiose (TC) was a gift from professor Helge Tollefsbøg (Nymoed Pharma A/S, Oslo, Norway). Na[125]I was from Amersham Pharmacia, Biotech (Little Chalfont, UK). 125I-TC-AOM was synthesized as described previously (29), mixed with 20 times as much unlabeled AOM, and added to cell suspensions at a final concentration of 200 nM, i.e. 10 μg/ml (specific activity, 20,000 dpm/μg of protein). Leupeptin was purchased from Protein Research Foundation (Osaka, Japan); 3-methyladenine was from Fluka A.G. (Buchs, Switzerland); Metrizamide, Nycodenz, and iodixanol were from Nycomed Pharma A/S, and Percoll was from Amersham Pharmacia Bio- tech (Uppsala, Sweden). Kits for automated analysis of lactate dehydrogenase (LDH) and acid phosphatase were obtained from Boehringer (Mannheim, Germany). Antibodies against superoxide dismutase, endosomum-associated protein 1, lysosomal glycoprotein Lgp120, and cathepsin B were kind gifts from Dr. Ling-Yi Chang (Duke University, Switzerland); Metrizamide, Nycodenz, and iodixanol were from Nycomed Pharma A/S. The densities of the fractions were calculated from the absorbance at 260 nm, corresponding to about 3 molecules of AOM/gold particle. The pellet was resuspended in 2 ml of buffered sucrose and recentrifuged, and the 125I step was omitted. In either case, the homogenate was cooled to 4 °C, and further purification was performed at this temperature. The nuclei were sedimented by centrifugation for 2 min at 4,000 rpm (2,000 × g) and washed once, and 14 ml of the combined postnuclear supernatants were placed on top of a discontinuous (two-step), isotonic Nycodenz gradient, containing 9.5% Nycodenz (in buffered sucrose diluted to 0.9% NaCl overnight) and 22.5% Nycodenz in 0.9% NaCl, (32, 33). The 17-ml upper layer was diluted with an equal volume of buffered sucrose and layered on top of a Percoll two-step gradient (21 ml of 33% Percoll in buffered sucrose in the upper layer; 7 ml of 22.5% Nycodenz in buffered sucrose in the lower layer) and centrifuged for 30 min at 20,000 rpm (72,000 × g) to remove the endoplasmic reticulum. The autophagic vacuoles were recovered as a 5-ml fraction from the lower part of the Percoll gradient (near the Nycodenz interface) and diluted with 3.5 ml of isotonic 60% iodoxanol, overlaid with 1.5 ml of 30% iodoxanol and 2.5 ml of buffered sucrose, and centrifuged for 30 min at 20,000 rpm (72,000 × g) to sediment the Percoll particles. The purified vacuoles (autophagosomes, autophagosomes plus amphisomes, or lysosomes) were pooled and resuspended in 4 ml of buffered sucrose for morphological or biochemical analysis.

**Preparation and Uptake of AOM-gold—AOM-gold, i.e. AOM conjugated to colloidal gold particles (5- to 10-nm diameter), was prepared according to established procedures (31, 32). Gold colloidal particles were mixed, at 60 °C, 160 ml of 0.125% gold chloride and 40 ml of tannic acid (0.125 or 0.0045%, for preparation of 3- and 10-nm gold particles, respectively) buffered with 0.2% trinatrium citrate dihydrate, keeping the mixture at 60 °C until a stable wine-red color had developed (in about 3 min). The volume was then diluted with an equal volume of buffered sucrose and layered on top of a discontinuous (two-step), isotonic Nycodenz gradient (near the Nycodenz interface) and diluted with 3.5 ml of isotonic 60% iodoxanol, overlaid with 1.5 ml of 30% iodoxanol and 2.5 ml of buffered sucrose, and centrifuged for 30 min at 20,000 rpm (72,000 × g) to remove the endoplasmic reticulum. The autophagic vacuoles were recovered as a 5-ml fraction from the lower part of the Percoll gradient (near the Nycodenz interface) and diluted with 3.5 ml of isotonic 60% iodoxanol, overlaid with 1.5 ml of 30% iodoxanol and 2.5 ml of buffered sucrose, and centrifuged for 30 min in a Beckman SW40 rotor at 20,000 rpm (72,000 × g) to sediment the Percoll particles. The purified vacuoles (autophagosomes, autophagosomes plus amphisomes, or lysosomes, depending on the pretreatment of the cells or the homogenate) floated to the iodoxanol/sucrose interface, where they could be recovered for morphological or biochemical analysis.

**Measurements of Radioactivity and Enzyme Activities—Acid-soluble and acid-insoluble 125I radioactivity was measured by γ-counting of gradient fractions after precipitation with 10% trichloroacetic acid (27). Acid phosphatase (β-glycerophosphatase) was determined according to Ames (36), and LDH was determined according to Bergmeyer (37), using a Technicon RA-1000 autoanalyzer for both assays. In some particularly important experiments, the gradient resolution of LDH was improved by subtracting, from each fraction, the background of LDH from disrupted hepatocytes measured in separate gradients as enzyme activity resistant to treatment of the cells with 10 mM of the autophagy inhibitor 3MA (19). Autophagic activity was measured in intact cells as the sequestration of cytosolic LDH into the sedimentable cell fraction.
Receptor-mediated endocytic uptake of AOM-gold. A, hepatocytes were incubated for 15 min at 37 °C with 125I-TC-AOM (○) or 125I-TC-AOM-gold (●) at the concentration indicated (about 3 molecules of AOM/gold particle). The amount of radioactivity taken up by the cells was measured and expressed as intracellular AOM concentration. B, hepatocytes were incubated for 2 h at 37 °C with 200 nM 125I-TC-AOM (○), 200 nM 125I-TC-AOM-gold (●), or 400 nM 125I-TC-AOM-gold (△) in the presence of unlabeled AOM at the concentration indicated. The amount of radioactivity taken up by the cells was measured and expressed as a percentage of the control value (in the absence of unlabeled AOM). Each panel represents a single experiment.

"cell corpses") following electrodissruption and expressed as a percentage of the total cell-associated LDH (2).

Protein Immunoblotting (Western Blotting)—Equal amounts of protein from the various organelle fractions (whole cell homogenates, lysosomes, autophagosomes, amphisomes + autophagosomes) or from a "residual fraction" prepared as autophagosomes, but from cells incubated with 3MA and homogenates treated with GPN (and applied as an autophagosome-equivalent volume rather than as an equivalent amount of protein), were subjected to one-dimensional SDS-polyacrylamide gel electrophoresis, using the mini-Protein equipment from Bio-Rad. The residual fraction was essentially devoid of autophagosomes, amphisomes, or lysosomes, thus serving to define the background of contamination by endoplasmic reticulum, endosomes, etc. in the or amphisomes, or lysosomes, thus serving to define the background of contamination by endoplasmic reticulum, endosomes, etc. in the or amphisomes, or lysosomes, thus serving to define the background of contamination by endoplasmic reticulum, endosomes, etc. in the organelle fractions. The separated proteins were transferred onto Nitro-pure nitrocellulose supports (Micron Separations Inc., West Borough, MA) using a Bio-Rad SemiDry Transblot apparatus. The blots were blocked overnight in 5% nonfat dried milk in Tris-buffered saline (TBS; 25 mM Tris, 0.8% NaCl, pH 7.4) and then incubated at room temperature for 2 h with primary antibody and for 30 min with horseradish peroxidase-conjugated secondary antibody (both in TBS with 2% dried milk). After each step, the blots were washed three times in TBS with 0.1% Tween 20. The antibodies were detected by chemiluminescence, using the SuperSignal peroxidase substrate (Pierce) and Kodak X-Omat LS films (Eastman Kodak Co.). The films were digitalized with a model 300A laser densitometer from Molecular Dynamics Inc. (Sunnyvale, CA).

Electron Microscopy—One ml of 3-nm AOM-gold suspension (absorbance of 0.1 at 520 nm after 100-fold dilution) was inoculated intravenously into rats 24 h before cell isolation to prelabel hepatic lysosomes. To label vacuoles of the endocytic-lysosomal pathway in isolated hepatocytes, the cells were incubated for 3 h at 37 °C with a low concentration of 10-nm AOM-gold (final absorbance of 2.0 at 520 nm, i.e. 1/50 of the concentration used for density shifting). The cells were washed once in wash buffer (30, fixed in 2% glacial acetic acid overnight at 4 °C, and postfixed for 60 min in 1% OsO4, reduced with 1.5% potassium ferricyanide, followed by en bloc staining with 1.5% uranylacetate. After serial dehydration in ethanol and propylene oxide, specimens were embedded in Epon and then sectioned and poststained with 0.2% lead citrate. Sections were examined in a Phillips CM10 electron microscope at 60 kV.

For quantitation of organellar numbers and gold contents, three blocks from each treatment group within each experiment were sectioned, and 2–4 cell profiles (with a nucleus) were examined on each grid. The number of endocytic and autophagic vacuoles per cell profile was counted in the microscope at ×13,500 magnification. For measurements of organellar diameters and volume fractions, 10 random micrographs for each treatment group were taken from four separate experiments (i.e. a total of 40 micrographs/treatment) and magnified to ×34,500, and the relative cytoplasmic volume fraction occupied by each type of organelle was determined morphometrically using a double lattice. Organellar profile diameters were generally recorded as the average of the shortest and longest diameter, except for small endocytic and autophagic vacuoles and vesicles, where the smallest diameter was used.

RESULTS

Receptor-mediated Uptake of AOM-gold by Isolated Rat Hepatocytes—AOM-coated 10-nm gold particles (AOM-gold), used in the present experiments both as an ultrastructural vacuole marker and as a perturbant of vacuole density, were taken up into isolated hepatocytes by a saturable process, like free AOM (Fig. 1A). The uptake of radiolabeled AOM-gold was competed out by unlabeled AOM with the same efficiency as was radiolabeled, free AOM (Fig. 1B), indicating that AOM-gold at the concentrations used (200–400 nM AOM) was taken up virtually exclusively by receptor-mediated endocytosis. The higher uptake efficiency of gold-bound AOM relative to free AOM in Fig. 1A probably relates to the fact that each gold particle carries about three molecules of AOM, which may be internalized by a single receptor engagement, as opposed to only one molecule of free AOM per receptor engagement.

Density Shifting of Hepatocytic Lysosomes and Autophagic Vacuoles by Endocytosed AOM-gold—Isolated rat hepatocytes were incubated at 37 °C under conditions of maximal autophagy (amino acid-free medium), with 125I-TC-AOM (200,000 dpm; 10 µg of protein/ml) included as a marker of endocytic-lysosomal vacuoles (26, 27). To shift the density of these vacuoles, colloidal 10-nm AOM-gold was added, replacing unlabeled AOM so as to leave the uptake of 125I-TC-AOM unaltered. After 3 h of endocytosis in the absence of AOM-gold, virtually all of the 125I-TC-AOM banded in a sucrose density gradient as a single peak at 1.19 g/ml, mostly in acid-soluble (i.e. degraded) form (Fig. 2A), at the same position as the lysosomal marker enzyme, acid phosphatase (Fig. 2B). When 10-nm AOM-gold was endocytosed along with the 125I-TC-AOM, nearly all of the acid-soluble and acid-insoluble radioactivity, as well as most of the acid phosphatase, sedimented to the bottom of the gradient tube (>1.20 g/ml), indicating that the lysosomes had become extensively density-shifted by fusion with AOM-gold-loaded endosomes.

Prelysosomal autophagic vacuoles (autophagosomes and amphisomes) can be recognized in density gradients by their contents of 3MA-sensitive, i.e. autophagocytosed, LDH (35), which becomes degraded once these vacuoles fuse with lysosomes (2). As seen in Fig. 2C, the peak of 3MA-sensitive LDL at 1.14 g/ml was only slightly shifted toward higher densities by endocytosed AOM-gold. This would suggest that in control cells, autophagocytosed LDL resides largely in autophagosomes rather than in amphisomes.

Elevation of Autophagocytosed LDL Levels by Late Stage Inhibitors of Autophagic Flux—Since so little prelysosomal LDL could be detected in control cells, an attempt was made to induce prelysosomal autophagic vacuole accumulation by incubating the cells with various inhibitors of late stages in the autophagic-lysosomal pathway (18, 35, 38). As shown in Fig. 3, the proteinase inhibitor leupeptin and the microtubule inhibitor vinblastine (Fig. 3A) as well as the amino acid asparagine and the lysosomotropic amines propylamine and ammonium chloride (Fig. 3B) induced 3MA-sensitive accumulations of LDL.

The Density Shifting of Lysosomes and Autophagic Vacuoles Is Inhibited by Vinblastine—Vinblastine inhibits microtubule-
and nuclear pellet. Shown represent the combined activities of the tube bottom sediment (including the last two fractions) and of material density-shifted to the fractions. The activity per fraction is given as a percentage of the corresponding total activity in the unfractionated disruptate. The three last values or 10-nm AOM-gold (10 mM 3-methyladenine and additional hepatocytes.

lysosomes to become acid-soluble (Fig. 4 accumulated in light (1.11 g/ml) and dense endosomes (1.14 g/ml) at 3 h, whereas very little radioactivity had reached the lysosomes to become acid-soluble (Fig. 4A, open symbols). The endosomes were extensively density-shifted upon co-endocytosis of AOM-gold (Fig. 4A, closed symbols), but the shift was less complete than in control cells, probably because vinblastine inhibits the overall endocytic uptake of AOM-gold (27).

Whereas the lysosomes from control cells banded as a sharp peak at 1.19 g/ml (Fig. 2B), the lysosomes from vinblastine-treated cells showed a broad density distribution between 1.13 and 1.19 g/ml (Fig. 2B). This distribution was virtually unaffected by AOM-gold, confirming the efficiency of vinblastine as an inhibitor of endosome-lysosome fusion (9, 40). Furthermore, the prelysosomal, LDL-containing autophagic vacuoles, accumulating to high levels in the presence of vinblastine (35), were also essentially unaffected by endocytosed AOM-gold under these conditions (Fig. 4C). The density shift experiment thus confirms the ability of vinblastine to prevent endosome-autophagosome fusion (9) and indicates that the autophagic vacuoles accumulating in the presence of vinblastine are autophagosomes rather than amphisomes.

dependent vacuole translocations, thereby preventing fusion between endosomes and lysosomes (27, 39, 40), between endosomes and autophagosomes (17), and between prelysosomal autophagic vacuoles and lysosomes (19, 41). In the presence of this drug, endocytosed 125I-Tc-AOM could be seen to have accumulated in light (1.11 g/ml) and dense endosomes (1.14 g/ml) at 3 h, whereas very little radioactivity had reached the lysosomes to become acid-soluble (Fig. 4A, open symbols). The endosomes were extensively density-shifted upon co-endocytosis of AOM-gold (Fig. 4A, closed symbols), but the shift was less complete than in control cells, probably because vinblastine inhibits the overall endocytic uptake of AOM-gold (27).

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Effect of Lysosomotropic Amines on AOM-gold-induced Density Shifts—Ammonia, propylamine, and other lysosomotropic, pH-elevating amines inhibit intralysosomal protein degradation and cause lysosomal swelling (42–45), possibly also accumulation of prelysosomal autophagic vacuoles (41). Both propylamine and ammonia (NH₄Cl) prevented the formation of acid-soluble degradation products from endocytosed 125I-Tc-AOM (Fig. 4, D and G) and made the lysosomes light, causing them to band broadly in the sucrose gradients with a peak around 1.15 g/ml (Fig. 4, E and H). Whereas the endocytosed 125I-Tc-AOM was extensively shifted (Fig. 4, D and G), AOM-gold induced only an insignificant lysosomal density shift (Fig. 4, E and H), suggesting that the amines had effectively prevented fusion between endosomes and lysosomes. Both amines caused extensive accumulation of autophagocytosed LDL (Fig. 4, F and I; compare with the control cells in Fig. 2C), presumably reflecting an inhibition of intralysosomal LDL degradation (18). Like the lysosomal marker enzyme acid phosphatase, the LDL peak was only slightly density-shifted by endocytosed AOM-gold (Fig. 4, F and I), compatible with an intralysosomal localization of the accumulated LDL.

Density Shifting of Endosomes, Lysosomes, and Autophagic Vacuoles in the Presence of Asparagine—Asparagine, an inhibitor of autophagic-lysosomal fusion that does not affect endocytic-lysosomal fusion (17–19), allowed endocytosed 125I-Tc-AOM to be density-shifted by endocytosed AOM-gold almost to
the same extent as in control cells (Fig. 5A). Unlike vinblastine and the lysosomotropic amines, asparagine also allowed extensive density shifting of lysosomes (Fig. 5B) and of the LDH-containing (prelysosomal) autophagic vacuoles that had accumulated in its presence (Fig. 5C); i.e. a significant fraction of latter were apparently amphisomes.
Leupeptin-induced Alterations in the Density Distribution and Density Shifting of Autophagic Vacuoles—Leupeptin, an inhibitor of lysosomal cathepsins (46) and of intralysosomal protein degradation (47, 48), reduces the ability of lysosomes to fuse with endosomes (49, 50) and with prelysosomal autophagic vacuoles (18), thereby causing accumulation of the latter (41). In cells incubated with leupeptin, endocytosed 125I-TC-AOM accumulated mainly in dense endosomes at 1.14–1.15 g/ml and was strongly density-shifted by AOM-gold (Fig. 5D), whereas the lysosomes at 1.19 g/ml were moderately density-shifted (Fig. 5E), suggesting a partial inhibition of endosome-lysosome fusion. Remarkably, the autophagocytosed LDH distributed as two distinct peaks: one dense peak coinciding with the lysosomal marker enzyme acid phosphatase activity (Fig. 5F). These two peaks would probably represent LDH inside degradation-suppressed lysosomes, and LDH in prelysosomal autophagic vacuoles, respectively (41). Endocytosed AOM-gold altered the LDH distribution dramatically, causing an extensive density shift of the light peak, with some of the LDH sedimenting to the bottom of the tube (Fig. 5F). A major fraction of the light autophagic vacuoles accumulating in the presence of leupeptin would thus appear to be amphisomes, acquiring a high density upon fusion between autophagosomes and heavy, AOM-gold-loaded endosomes.

Electron microscopic examination, using AOM-gold particles (loaded at moderate quantities so as to avoid a density shift) to mark the amphisomes, confirmed that autophagosomes and amphisomes were present in approximately equal numbers in the light (1.14 g/ml) peak fraction (51% autophagosomes, 43% amphisomes, 6% lysosomes), whereas autophagosomes predominated in the preceding (1.13 g/ml) fraction (80% autophagosomes, 19% amphisomes, 1% lysosomes) and lysosomes in the following (1.15 g/ml) fraction (14% autophagosomes, 36% amphisomes, 50% lysosomes). An amphisome from the light peak fraction is shown in Fig. 6H.

Leupeptin-induced Alterations in the Distribution and Contents of Endocytic and Autophagic Vacuoles—In an ultrastructural study, hepatocytic lysosomes were prelabeled in vivo overnight by an intravenous injection of 3-nm AOM-gold, whereas the endocytic pathway was labeled by a 3-h uptake of 10-nm AOM-gold into the subsequently isolated hepatocytes. Leupeptin did not affect the overall endocytic uptake of 125I-TC-AOM-gold (results not shown), and the number of gold particles per cell profile was similar in leupeptin-treated and control cells (810 ± 219 and 724 ± 131, respectively; mean ± S.E. of five independent experiments). The sizes of endocytic and autophagic vacuoles were also unaffected by leupeptin according to morphometric/ultrastructural studies (vacuole diameters, in nm, in control and leupeptin-treated cells, respectively, as follows: endocytic vesicles and tubules, 78 ± 7 and 74 ± 5; multivesicular endosomes, 334 ± 15 and 360 ± 17; autophagosomes, 787 ± 40 and 806 ± 42; amphisomes, 822 ± 37 and 873 ± 48; lysosomes, 1,141 ± 63 and 1,342 ± 98 (mean ± S.E. of 22–44 organelle profiles)).

One 10-nm gold-labeled endocytic vacuole category was represented by small endocytic vesicles and tubules, about 70 nm in diameter, usually found in the periphery of the cell (Fig. 6A). These are thought to be extensively interconnecting elements of an early, tubulovesicular endocytic compartment (26, 51). The vesicle profiles contained only one or two gold particles each, implying that a significant fraction of them probably remained unlabeled.

Another morphologically recognizable vacuole category was the multivesicular endosomes, 300–400 nm in diameter (Fig. 6B). The smaller of these may still be connected to the peripheral tubulovesicular network, whereas the larger ones are mostly detached, late endosomes en route to the lysosomes (26, 27), from which they could be distinguished by the absence of 3-nm gold. The multivesicular endosomes (B) are seen to contain 10-nm gold. Autophagosomes (C) are gold-negative vacuoles with a content of undegraded cytoplasm, delimited by double or multiple membranes. Lysosomes (D) are labeled with 3-nm gold (small arrowhead) and often with 10-nm gold (large arrowhead) as well and contain more or less degraded material of autophagic or endocytic origin. Amphisomes, from control (E) or leupeptin-treated cells (F, G), are autophagic-endocytic fusion vacuoles that contain both endocytosed 10-nm gold (large arrowhead) and undegraded cytoplasm but no 3-nm gold particles. In the amphisome in F, the two endocytic fusion partners can be recognized as multivesicular endosomes. Amphisomes recovered from sucrose gradients (H) or from a preparation of purified autophagic vacuoles (I and J) have the same morphology as in intact cells, containing autophagocytosed material and endocytosed 10-nm AOM-gold (large arrowhead). Magnification, × 32,000 (bar, 100 nm).
Isolated rat hepatocytes, containing lysosomes prelabeled with 3-nm AOM-gold by an intravenous injection 24 h before cell isolation, were incubated for 3 h at 37 °C with 10-nm AOM-gold in the presence or absence of leupeptin (0.3 mM). After incubation, the cells were fixed and processed for conventional electron microscopy. The number of autophagic and endocytic organelle profiles and the number of gold particles in each of the categories listed were counted in four or five independent experiments, representing 40–50 cell profiles within each treatment group. Among the endocytic vacuoles (multivesicular endosomes or small tubules/vesicles), only those containing 10-nm gold were counted. Among the autophagic vacuoles (including the lysosomes), both vacuoles with and without gold particles were counted. Each value represents the mean ± S.E. of four or five independent experiments (specified in parentheses).

extrapolated to 0, confirmed that the expected fraction of false negative amphisomes was only about 6% (results not shown).

The hepatocytic lysosomes included a few small dense bodies (presumably resting lysosomes), but under the present conditions, with maximal autophagy, the majority were large, electron-lucent vacuoles that contained autophagocytosed cytoplasm at various stages of degradation (Fig. 6D). Most of the lysosomes were labeled with recently endocytosed 10-nm gold, demonstrating their simultaneous engagement in both endocytic and autophagic degradation (9). Leupeptin treatment had little effect on the numbers of endosomes and 3-nm gold-labeled lysosomes but induced a significant accumulation of autophagosomes and 3-nm gold-negative, 10-nm gold-positive autophagic vacuoles (Table I). The latter represent a mixture of amphisomes and 3-nm gold-negative lysosomes, only about one-half of the lysosomes being labeled by 3-nm AOM-gold overnight in vivo (26). Assuming that 3-nm gold-positive and -negative lysosomes are similarly unaffected by leupeptin, the increase within the 3-nm gold-negative vacuole class would all be due to amphisome accumulation. Volume fraction changes closely followed the changes in vacuole numbers (according to a morphometric analysis of 40 micrographs from four independent experiments; results not shown), e.g. with a highly significant 130% leupeptin-induced increase in the volume fraction of the 3-nm gold-negative amphisomes/lysosomes. Furthermore, a 10-nm gold particle count, indicative of endocytic flux, revealed that whereas leupeptin had no effect on gold flux through the endocytic compartments, it caused an accumulation of gold particles in the mixed amphisome/lysosome class at the expense of the pure (3-nm gold-positive) lysosomes (Table I). Leupeptin thus clearly interfered with the flux of endocytosed AOM-gold from amphisomes to lysosomes.

**TABLE I**

| Vacuole category                      | No. of organelle profiles/cell profile | Percentage of 10-nm gold particles in each vacuolar compartment |
|---------------------------------------|---------------------------------------|---------------------------------------------------------------|
|                                       | Control With leupeptin                | Control With leupeptin |
| Small endocytic tubules/vesicles      | 9.6 ± 1.4 (5)                         | 14.9 ± 2.6                                     |
| Multivesicular endosomes              | 8.2 ± 1.2 (5)                         | 10.4 ± 1.4                                     |
| AVs without gold (autophagosomes)     | 3.6 ± 0.2 (4)                         | 8.3 ± 1.0                                     |
| AVs with 3-nm gold (lysosomes)        | 5.5 ± 0.8 (4)                         | 5.3 ± 0.9                                     |
| AVs with 10-nm gold only (amphisomes + lysosomes) | 4.8 ± 0.8 (4) | 12.2 ± 1.4                                     |

*p < 0.005 for significance of difference versus control according to Student’s t test.

b *p < 0.02.

**Fig. 7.** Protein immunoblotting (Western blotting) of organelle markers. Hepatocytes were incubated for 3 h at 37 °C with 0.3 mM leupeptin (for preparation of amphisome-enriched autophagic vacuoles, Amphisomes + autophagy), or with 50 μM vinblastine (for preparation of all other fractions). Subcellular fractions were prepared as described under “Experimental Procedures,” and equivalent amounts of protein from the various organelle fractions were subjected to SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes. The cytosolic marker protein superoxide dismutase (SOD), the lysosomal glycoprotein Lgp120, the lysosomal proteinase cathepsin B, the early endosome-associated ASGPR, the early endosome-associated protein 1 (EEA1), and the late endosome-associated MPR were detected with specific antibodies and a peroxidase/chemiluminescence staining method.

The stable cytosolic marker, superoxide dismutase, is delivered to autophagic vacuoles through autophagy (52) and was, accordingly, present in all fractions (except in the residual fraction). The lysosomal markers, cathepsin B and the membrane glycoprotein Lgp120, were present virtually exclusively in the lysosomes. Two early endosome markers, the ASGPR and the early endosome-associated protein 1 (EEA1), were clearly present in the amphisome-enriched vacuole preparation but hardly detectable in autophagosomes or lysosomes. The same was true for a late endosome marker, the MPR. Both early and late endosomes would thus seem able to fuse with autophagosomes to form amphisomes. The endosomal contamination of these fractions was negligible, as indicated by the lack of mark-
ers in the autophagy-suppressed residual fraction (results from vinblastine-treated and leupeptin-treated cells being similar). The lack of endosomal markers in the lysosomes indicates, furthermore, that the markers are either rapidly degraded there or (more likely, since e.g. ASGPR is metabolically stable) recycle rapidly from amphisomes or lysosomes.

**DISCUSSION**

The present results demonstrate that amphisomes can be distinguished physically (in sucrose density gradients and by purification) as well as morphologically (in the electron microscope) from other, closely related autophagic and endocytic vacuoles. Sucrose density gradients have previously been shown to be suitable for the separation of hepatocytic endosomes from lysosomes (54), whereas prelysosomal autophagic vacuoles, accumulating in the presence of vinblastine, have been found to band in these gradients at the same position as the lysosomes (35). In the present study, the two types of vacuole could be seen to have overlapping gradient distributions not only after vinblastine treatment but also after treatment with other autophagic flux inhibitors such as propylamine, ammonia, or asparagine, all of which induced autophagic vacuole accumulation as previously observed (2, 18). Prelysosomal autophagic vacuoles from control cells were lighter than lysosomes, but these vacuoles are rather short lived (55), and their number at steady state is low even when autophagy is running at its maximal rate (2, 18). The small peak of LDH-containing autophagic vacuoles from control cells was only slightly density-shifted in the sucrose gradients by endocytosed AOM-gold, suggesting that few amphisomes were present. However, the vacuole numbers recovered were too small to permit meaningful quantitative estimates on the basis of the density shift.

Leupeptin, an inhibitor of intralysosomal proteolysis (47, 48), has previously been shown to induce the development of congested, protein-filled lysosomes as well as a secondary accumulation of prelysosomal autophagic vacuoles (41, 56), reflecting a transient inhibition of autophagic flux into the lysosome (18). In isotonic density gradients, the congested lysosomes were found to have a higher density than normal lysosomes (57), but since leupeptin increases the density of prelysosomal autophagic vacuoles as well, the latter could not be separated from lysosomes in isotonic metrizamide density gradients (58). The presently observed banding of prelysosomal autophagic vacuoles from leupeptin-treated cells as a distinct, light peak in hypertonic sucrose density gradients was, therefore, a pleasant surprise. One possible explanation is that autophagosomes and amphisomes (and multivesicular endosomes as well) contain vesicles with intact membranes that may, under hypertonic conditions, better resist the osmotic extraction of water than do the lipolytically degraded membrane structures inside the lysosomes. Although the criteria used to define prelysosomal autophagic vacuoles in the gradients (presence of LDH, lack of acid phosphatase) could not distinguish amphisomes from autophagosomes, the extensive density shifting of the prelysosomal peak by endocytosed AOM-gold demonstrates unequivocally that the majority of these vacuoles are amphisomes, containing both autophagocytosed (LDH) and endocytosed (AOM-gold) material. AOM-gold-induced density shifting is thus a powerful method, which may provide a valuable supplement to the established diaminobenzene density shift technique (59).

The present ultrastructural, morphometric analysis, using endocytosed AOM-gold to mark the amphisomes, confirms our gradient data in suggesting that the majority of the prelysosomal autophagic vacuoles accumulating in the presence of leupeptin were indeed amphisomes. Furthermore, our quantitative analysis of the endocytic flux of AOM-gold clearly showed a leupeptin-induced accumulation of gold particles in the amphisomes at the expense of the lysosomes, thus providing additional evidence for an inhibition of amphisome-lysosome fusion by leupeptin. Somewhat surprisingly, leupeptin did not induce any detectable retention of AOM-gold in the late, multivesicular endosomes, despite the documented ability of the drug to inhibit endosome-lysosome fusion (49). A likely explanation would be that the “endosomes” that accumulate degraded 125I-TC-AOM in the presence of leupeptin are actually amphisomes, banding at the same sucrose gradient density (1.14 g/ml) as the late endosomes (26). Perhaps leupeptin inhibits only that part of the endocytic flux that passes via the amphisomes, leaving any direct endosome-lysosome fusion unaffected. The fusion activity of the autophagosomes, on the other hand, is probably inhibited by leupeptin, as suggested by the 2-fold accumulation of autophagosomes seen in the presence of the drug, but it is not clear whether this represents a primary inhibition of autophagosome-lysosome fusion or reduced autophagosome-amphisome fusion, e.g. as a secondary consequence of impaired amphisome-lysosome fusion (cf. Fig. 8 for overview).

Our electron micrographs indicate that amphisomes may undergo multiple independent fusions with multivesicular endosomes, at different places on the amphisome surface. Multivesicular endosomes can be either early (ligand-recycling, attached to the peripheral tubulovesicular endocytic network) or late (free endosomes without ligand recycling) (26, 27). The fact that amphisomes (but not autophagosomes or lysosomes) contain the MPR, generally regarded as a marker of late endo-
somes (60), suggests that they have undergone fusion with late endosomes. However, the amphisolal localization of typical early endosome markers like the asialoglycoprotein receptor (21) and the early endosome-associated protein 1 (53) indicates that even relatively early (sorting) endosomes may fuse with autophagosomes and amphisomes (Fig. 8). The possibility should probably be considered, however, that the transition from early to late endosomes may represent a very gradual maturation process (60) and that, in hepatocytes at least, the endosomes fusing with autophagosomes/amphisomes may be free multivesicular endosomes carrying a variable mixture of early and late endosome markers. The total absence of endosome markers in the lysosomes indicates that the markers are either rapidly degraded in the lysosomes or (more likely, since e.g. ASGPR is metabolically stable) recycle rapidly from amphisomes or lysosomes. Hepatocytes thus present a clean cut distinction between MPR-positive, Lgp120-negative prelysosomal vacuoles (endosomes and amphisomes) and MPR-negative, Lgp120-positive lysosomes (9). In other cell types, early lysosomes may contain MPR (61), and under some conditions all of the lysosomes may be MPR-positive (22).

Endosomes are apparently capable of fusing directly with lysosomes as well as with autophagosomes/amphisomes (6, 12, 19, 21, 24, 25), but they seem to have a 5–6-fold higher propensity to fuse with autophagosomes or amphisomes (21). The inability of some authors to observe fusion between endosomes and prelysosomal autophagic vacuoles (24, 25) may relate to the definitions, markers, and methods used. Amphisome formation has been demonstrated in several nonhepatic cells and tissues (22, 23) and is likely to be a general biological phenomenon. Amphisomes will eventually fuse with small lysosomes, probably independently of endosomes, as indicated by the separate attachments of lysosomes and endosomes to the surface of amphisomes from leupeptin-treated cells. Several small lysosomes can apparently attach to, and fuse with, the same autophagic vacuole to form large, active lysosomes, accounting for lysosomal heterogeneity and for the gradual increase in lysosomal hydrolytic capacity (52). Secondary lysosomes with autophagic contents are often described as “autolysosomes,” but since all lysosomes are capable of simultaneous engagement in autophagy and endocytosis (9, 62), the term “active lysosomes” may be more appropriate (to be contrasted with the “passive,” unengaged lysosomes of the dense body type; cf. Refs. 58 and 63). It is also possible that amphisomes may receive some lysosomal enzymes (albeit apparently not cathepsin B) through the endocytic pathway. Certain lysosomal enzymes, such as acid phosphatase, are synthesized as transmembrane proteins that enter the endocytic pathway (via the plasma membrane) due to specific sorting motifs in the cytoplasmic tail of the enzyme (51, 64, 65). Other lysosomal enzymes are bound to mannose 6-phosphate receptors in the trans–Golgi network (66, 67) and brought to a late endosomal compartment, directly or via the plasma membrane, by the help of similar endocytic sorting motifs in the receptor molecule (51). Since amphisomes are acidic (12, 20), probably due to a proton pump brought in by the endosomal fusion partner (68), the possibility cannot be excluded that limited proteolytic activity may take place in the amphisomes. Morphological evidence indicates that autophagic inputs to amphisomes and lysosomes can also be multiple (Ref. 52 and present data). Biochemical studies have suggested that the amphisomes exhibit a certain permanence and are able to receive a continued autophagic influx even if the endocytic influx is blocked (19). Conversely, endocytic influx to amphisomes as well as to lysosomes can apparently proceed in the absence of autophagy (19), and direct fusion between lysosomes and late endosomes has been observed under cell-free conditions (69). There is, furthermore, strong morphological evidence for direct fusion of autophagosomes with lysosomes (6, 12, 24, 25). In addition to these various heterotypic fusions, the lysosomes are known to undergo frequent homotypic fusions and fissions, with extensive exchange of contents (70, 71), and late endosomes have been shown to be capable of homotypic fusion under cell-free conditions (72). Autophagosomes, multivesicular endosomes, amphisomes, and lysosomes seem, therefore, to be rather promiscuous in their choice of fusion partners, although some preferences can be discerned (21).

In the original biochemical studies that identified the amphisome, asparaginase was used, as a selective inhibitor of amphisome-lysosome fusion, to induce accumulation of LDH-containing amphisomes (17). The present study confirmed the ability of asparaginase to induce accumulation of autophagocytosed LDH and of LDH-containing vacuoles. Although the latter could not be resolved from lysosomes in sucrose density gradients, they were extensively density-shifted by endocyto.sed AOM-gold, unlike the LDH-containing lysosomes from ammonia- or propylamine-treated cells, thus discounting the possibility that the asparagine effect could be mediated by its deamination to ammonia, a lysosomotropic agent (43, 44). Asparagine (but not ammonia or propylamine) likewise allowed density shifting of endosomes and lysosomes, supporting the notion that the latter were not detectably affected by asparagine-generated ammonia. The gradient data are thus consistent with the hypothesis that LDH accumulates in amphisomes after asparaginase treatment and in lysosomes after ammonia or propylamine treatment (18).

Microtubule inhibitors like vinblastine and colchicine block both the endocytic and the autophagic flux in isolated rat hepatocytes (39, 73) and cause accumulation of endosomes and prelysosomal autophagic vacuoles (9, 41, 74). We have recently demonstrated that vinblastine inhibits the transfer of endocyto.sed AOM to late endosomes by preventing the microtubule-dependent maturation of multivesicular endosomes from the early tubulovesicular network (26, 27). The present results show that the density of the endosomes could still be shifted by AOM-gold in the presence of vinblastine, but the density distributions of autophagic vacuoles and lysosomes remained unaffected. Vinblastine thus appears to effectively block endocytic influx to the autophagic pathway. Its effect within the autophagic pathway is apparently to inhibit the fusion of autophagosomes and amphisomes with lysosomes, probably without affecting autophagosome-amphisome fusion (19). However, since no new amphisomes are formed, the net result of vinblastine treatment is an extensive accumulation of autophagosomes, which has been utilized in attempts to purify these organelles (35).

In conclusion, the present study shows that amphisomes are distinct organelles that can be separated from other autophagic vacuoles, using leupeptin to prevent their fusion with lysosomes and using endocyto.sed AOM-gold to density-shift them away from autophagosomes. Amphisomes form by single or multiple autophagosome-endosome fusions and can be recognized morphologically by their mixed autophagic-endocytic contents. Novel procedures for purification of autophagosomes and partial purification of amphisomes have allowed a preliminary biochemical characterization of both organelles. Amphisomes lack lysosomal marker enzymes (acid phosphatase and cathepsin B) and lysosomal membrane proteins (Lgp120) but may carry both early and late endosomal markers like the asialoglycoprotein receptor, the early endosome-associated protein 1, and the cation-independent mannose 6-phosphate receptor.

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Rat Liver Amphisomes 21891

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