The Autophagic and Endocytic Pathways Converge at the Nascent Autophagic Vacuoles

Willisa Liou,*§ Hans J. Geuze,* Math. J.H. Geelen,‡ and Jan W. Slot*

*Department of Cell Biology, Faculty of Medicine, †Department of Biochemistry, Faculty of Veterinary Medicine, Institute of Biomenbranes, Utrecht University, 3584 CX Utrecht, The Netherlands; and ‡Department of Anatomy, Chang Gung College of Medicine and Technology, Taiwan, Republic of China

Abstract. We used an improved cryosectioning technique in combination with immunogold cytochemistry and morphometric analysis to study the convergence of the autophagic and endocytic pathways in isolated rat hepatocytes. The endocytic pathway was traced by continuous uptake of gold tracer for various time periods, up to 45 min, while the cells were incubated in serum-free medium to induce autophagy. Endocytic structures involved in fusion with autophagic vacuoles (AV) were categorized into multivesicular endosomes (MVE) and vesicular endosomes (VE). Three types of AV—initial (AVi), intermediate (AVi/d), and degradative (AVd)—were defined by morphological criteria and immunogold labeling characteristics of marker enzymes.

The entry of tracer into AV, manifested as either tracer-containing AV profiles (AV+) or fusion profiles (FP+) between AV and tracer-positive endosomal vesicles/vacuoles, was detected as early as 10 min after endocytosis. The number of AV+ exhibited an exponential increase with time. FP+ between MVE or VE and all three types of AV were observed. Among the 112 FP+ scored, 36% involved VE. Of the AV types, AVi and AVi/d were found five to six times more likely to be involved in fusions than AVd. These fusion patterns did not significantly change during the period of endocytosis (15–45 min). We conclude that the autophagic and endocytic pathways converge in a multistage fashion starting within 10 min of endocytosis. The nascent AV is the most upstream and preferred fusion partner for endosomes.

The autophagic and endocytic pathways represent branches of the lysosomal digestive system, the former being responsible for degradation of cytoplasmic constituents and the latter being responsible for degradation of exogenous substances. Although there is no direct evidence to disprove that autophagic and endocytic pathways can indeed run in parallel, both biochemical and morphological evidence has supported the convergence view (Høyvik et al., 1987; Rabouille et al., 1993).

The formation of the autophagic vacuoles (AV) begins with segregation of portions of cytoplasm by a lipid-rich membranous apparatus called phagophore (Seglen, 1987), generating nascent or immature autophagic vacuoles (AVi). Through acquisition of lysosomal membrane proteins, acidic pH, and hydrolases, the AVi becomes degradative (AVd) (Dunn, 1990, 1994). Its limiting membrane becomes one of uniform width and the contents appear to be disintegrating. From the standpoint of autophagy, the classical view has been that AV fuse with preexisting lysosomes (Aristila and Trump, 1968) while receiving acid hydrolases and encountering exogenous materials. That is, the autophagic and endocytic pathways converge at the lysosomal level. However, the biochemical study of carbohydrate degradation by Gorden and Seglen (1988) provided the first indication that AV might encounter endosomes before undergoing transformation through fusion with lysosomes: when autophagic–lysosomal fusion was blocked by asparagine, autophagocytosed [14C]lactose could still be degraded by endocytosed β-galactosidase. The term “amphisome” was then proposed for this functional compartment where the two pathways first meet and wherein endogenous β-galactosidase, a lysosomal marker enzyme, was missing. Later, amphisomes were proved to be acidic (Strømhaug and Seglen, 1993).

During the past decades, efforts to unravel the biogenesis of lysosomes have revealed that endosomes are also endowed with lysosomal characteristics, including lysosomal...
membrane proteins and lytic activities. Using the man-
nose-6-phosphate receptor (MPR) as a prelysosomal
marker, several immunocytochemical studies have docu-
mented the presence of MPR in AV (Dunn, 1990b; Tooze
et al., 1990; Punnonen et al., 1992, 1993). Although sub-
stantiating the view of prelysosomal convergence, these
studies did not provide morphological proof of the exist-
ence of amphisomes because a significant amount of lyso-
sumal enzymes was always present together with MPR in
the same autophagic compartment.

Also not clear is whether the coalescence of AV with ei-
ther lysosomes or endosomes is a random event or instead
exhibits (temporal) preferential fusion combinations.
Lawrence and Brown (1992) used a double-pulse labeling
strategy to separately mark lysosomes and endosomes
in an effort to determine which compartment initially
merges with AV. They found that soon after the induction
of autophagy and the onset of endocytosis, the preloaded
lysosomal tracers readily labeled the AVd, whereas AVi
received neither tracers even after 2 h. Yokota and col-
leagues (1995) approached the issue by quantifying the fu-
son profiles (FP) between different types of endosomes
and AV. Similar to Lawrence and Brown (1992), they used
leupeptin to deter the disappearance of AV. The most fre-
cently (64.2%) observed fusion occurred between lysoso-
mal marker–positive AV (i.e., AVd) and late endosomes,
but they did observe fusions between newly formed AV
and late endosomes, though only in rare cases (Yokota
et al., 1995).

In an attempt to trace the earliest point of convergence
between autophagic and endocytic pathways, Tooze and col-
leagues (1990) used HRP as an endocytic tracer in
CoCl2-treated exocrine pancreatic cells. They found that
Type I AV, the intermediate AV type (AVi/d) in their
classification, were first accessed by HRP after a 30-min
lag. Although FP as such were not studied, these authors
indirectly suggested that the endocytic and autophagic
pathways converge immediately after the formation of the
first compartments, i.e., early endosomes and nascent AV,
of each pathway, respectively.

The mainstay of our current study has been to follow the
delivery of interiorized tracer to AV. A time-sequential
FP analysis in the absence of drugs has been chosen to
resolve the issue of convergence. We asked: (a) How soon
after the endocytosis can convergence be detected? (b) Is
the convergence of single-point or multistage nature? (c)
If fusion takes place at various levels of each pathway,
does it occur to the same extent or do preferred sites exist?
We have used an improved cryosectioning technique that
greatly enhanced the fine structural preservation of AV
(Liou et al., 1996). Not only were fusion profiles better re-
tained structurally, but the morphometric analysis and the
designation of AV maturation stages, as defined before
(Rabouille et al., 1993), were also greatly facilitated. Our
data indicate that the autophagic pathway coalesces with
the endocytic pathway as soon as the AVi are formed.

Materials and Methods
Chemicals and Equipment

Obtained from Boehringer Mannheim Corp. (Indianapolis, IN): protein
A; from Dulbecco: Dulbecco’s minimal essential medium (DMEM, Gibco,
Paisley, UK); from Fluka (Buchs, Switzerland): polyvinylpyrrolidone (PVP,
med wt 10,000; from Sigma Chemical Co. (St. Louis, MO): methyl cellulose
25 cP; from Leica (Vienna, Austria): Reichert ultratract S/Reichert FCS
equipped with an antistatic device from Diatome (Biel, Switzerland); and
from Drukker International (Cuijk, The Netherlands): diamond knives.

The Preparation of Protein A Gold Conjugates

Colloidal gold solutions were prepared by the tannic acid reducing
method. We used H2O2 to oxidize the residual tannic acid after the gold
solution was formed (Slot and Geuze, 1985). H2O2 was added to a final
concentration of 0.2% to the gold solution after the reducing reaction was
complete. It was boiled for another 5 min. Protein A was added to this
gold solution to make complexes (Roth et al., 1978). 5-nm gold particles
were used as endocytic tracers, whereas 10- and 15-nm gold particles were
used on cryosections as immunolocalization markers. The H2O2 reduction
and the choice of protein A to stabilize the gold for uptake experiments
reduced the stickiness of the endocytosis marker to the surface of the liv-
ing cells, thus providing an uncompromised fluid-phase marker.

Animals, Cells, and Incubation

Four male Wistar rats weighing 200–250 g were fasted overnight and their
hepatoocytes were isolated according to Seglen (1976) with modifications
described previously (Geelen et al., 1978). The cells were suspended in
DMEM supplemented with 10% FCS and plated, 3 × 105 cells each, on
6-cm diameter collagen-coated, vented culture dishes. After 15 min of set-
tlement at room temperature, the cells were transferred into a 37°C chan-
er with a gas phase of 5% CO2/95% air and incubated for 1 h. The
DMEM was then replaced with suspension buffer (in mg/1,000 ml of final
solution: NaCl, 4,000; KCl, 400; CaCl2·2H2O, 180; MgCl2·6H2O, 130;
KH2PO4, 150; Na2SO4, 100; Hepes, 7.200; TES, 6.900; Tricine, 6.500;
and 52.5 ml of 1 M NaOH, pH 7.6; Seglen, 1976) to induce autophagy for at
least another hour before commencement of tracer uptake.

Endocytosis of Gold Tracer

Small gold was dialyzed against 0.9% NaCl for at least 2 h before use. It
was diluted in suspension buffer to a final OD280nm of 5. In the hepatocyte
cultures, the plain suspension buffer was replaced by a gold-containing one,
in such a way that at the end of an endocytosis experiment, all the cells
were starved for 1.75 h in suspension buffer but had been exposed to tracer
for 10 to 45 min. Endocytosis was terminated by fixation with 0.8% glutaral-
dehyde, 0.5% acrolein buffered at pH 6.3 according to Small and Cells (1978).

Ultracytotomy and Immunocytochemistry

After 1 h of fixation at room temperature, the cells were rinsed with 0.1 M
Pipes, pH 7.2, scraped off from the plate using a rubber policeman, trans-
ferrred into Eppendorf tubes, and centrifuged. Cell pellets were then resus-
pended in 10% gelatin and pelleted again. The tubes were plunged into
icy water to quickly solidify the gelatin with cells. In a cold room, the tip of
the Eppendorf tube was cut open and the cell pellet retrieved into a drop of
PVP-sucrose (15%/1.7 M), in which the pellet was cut into 1-mm
blocks. It was boiled for another 5 min. Protein A was added to this
solution to oxidize the residual tannic acid after the gold
reduction
reaction was complete. It was boiled for another 5 min. Protein A was added to this
gold solution to make complexes (Roth et al., 1978). 5-nm gold particles
were used as endocytic tracers, whereas 10- and 15-nm gold particles were
used on cryosections as immunolocalization markers. The H2O2 reduction
and the choice of protein A to stabilize the gold for uptake experiments
reduced the stickiness of the endocytosis marker to the surface of the liv-
ing cells, thus providing an uncompromised fluid-phase marker.

Quantitations

AV/Cell and AVi/AV. Profile counting was directly performed in an elec-
tron microscope (model 1200 EX; JEOL, Tokyo, Japan) operating at 60
kV using an objective aperture of 20 μm. Cell profiles were randomly cho-
sen and the number of AV profiles therein scored. The profile number of
AV per cell (AV/cell) was averaged from seven time points studied (441
cells in total). Sections from all seven time points were likewise randomly

The Journal of Cell Biology, Volume 136, 1997 62

62
Percentage of AV types in fusion 63.0
Relative volume density 34.9
Relative surface density 33.9

Table I. Endosome–AV Fusion Preference

| Row | Category                                      | AVi | AVi/d | AVd |
|-----|----------------------------------------------|-----|-------|-----|
| 1   | Percentage of AV types in fusion profiles    | 63.0 ± 3.6 | 18.1 ± 2.3 | 18.9 ± 2.3 |
| 2   | Percentage of AV types encountered           | 30.2 ± 2.7 | 9.0 ± 0.7 | 60.8 ± 3.0 |
| 3   | Relative volume density of AV types          | 34.9 ± 2.9 | 9.8 ± 0.5 | 55.3 ± 3.1 |
| 4   | Relative surface density of AV types         | 33.9 ± 3.9 | 10.0 ± 0.5 | 56.1 ± 4.0 |
| 5   | Fusion preference factor                     | 5.5  | 5.4   | 1.0  |

Row 1: averaged from data given in Fig. 7 (n = 7 ± SEM).
Rows 2, 3, and 4: results of morphometric analysis as described in Materials and Methods (n = 5 ± SEM).
Row 5: calculated as the ratio of values in row 1/values in row 4, normalized to the value of AVd set as 1.

Results

Characterization of AV Types

The cryosection is a most favorable substrate for immuno-electron microscopy. However, in its application to the study of autophagy, this advantage is offset by the lack of structural integrity of AV (Fig. 1a). A morphometric analysis of AV parameters, in particular, has been extremely cumbersome (Punnonen et al., 1992; Rabouille et al., 1993; Fersgnud et al., 1995). Recently, we have overcome this obstacle by replacing the conventional section retrieval medium, sucrose, by MU (Liou et al., 1996). This modification greatly improves the preservation of AV and allows reliable recognition of structural details in defining AV types (Fig. 1b). We tentatively defined three types of AV by morphological criteria as follows: (1) AVi are surrounded by multiple membranes and contain unaltered cytoplasmic constituents. Probably because of the scarcity of proteins and enrichment of lipids (Reunanen et al., 1985), the enveloping membranes of AVi are often indistinctive. These characteristics together with the presence of electronlucent material between the sequestered contents and the surrounding cytoplasm were used as convenient diagnostic features for AVi. (2) AVd are enclosed by a single distinctive membrane and contain components at various stages of degradation. This category also includes those AV exhibiting electron-opaque, homogenous matrices (i.e., residual body type of lysosomes). (3) AVi/d have a single limiting membrane and their contents show no (or only early) signs of degradation.

To validate these tentative morphological criteria of AV classification, we first studied the immunolabeling patterns of SOD, CAIII, and cathepsin D in the improved cryosections. In a previous study, we defined AV types using the presence of SOD and CAIII as discriminating immunocytochemical markers (Rabouille et al., 1993). Both are cytosolic enzymes and are randomly autophagocytosed into AV. The AVi exhibit SOD- and CAIII-labeling densities similar to that of the adjacent cytosol. In AVi/d, CAIII labeling is less than in the cytosol because of degradation, whereas the SOD, being degradation resistant, is slightly concentrated. The AVd show hardly any CAIII but do show dense labeling of SOD. In the present study, we first used a quantitative approach to verify the SOD-labeling pattern. In each of five cell preparations, ~100 AV profiles were randomly selected and classified solely on the basis of the above morphological criteria. Subsequently, the vacuolar versus cytosolic SOD-labeling densities of each AV type was determined. The SOD concentration ratios (± SEM) in AVi, AVi/d, and AVd over the cytoplasm were 1.39 ± 0.15, 2.35 ± 0.13, and 4.29 ± 0.67, respectively. These values are in good agreement with those reported before from rat liver tissue (Rabouille et al., 1993). In addition, the immunolabeling features of cathepsin D (Fig. 2a) and CAIII (Fig. 2, b and c) in the three AV types were in accordance with the earlier observations (Rabouille et al., 1993). Therefore, in the following experiments, we used only the morphological criteria to define AVi, AVi/d, and AVd. Cathepsin D labeling was low or absent in AVi and low in AVi/d. Virtually all AVd showed high labeling for cathepsin D (Fig. 2a) and were the exclusive sites where significant cathepsin D labeling was observed. The same holds for another lysosomal marker, LGP-120 (not shown). Since all lysosomes showed high SOD labeling, it seems that all lysosomes are involved in autophagy.
**Entrance of Endocytosed Tracers into AV**

As early as 10 min after endocytosis, AV⁺ profiles were detected, suggesting that the convergence of the two pathways starts within 10 min of endocytosis. Since the incidence was low, i.e., <1 per 100 cell profiles, the AV⁺ profiles counted after 10 min of uptake would not appear in graphic data. The occurrence of AV⁺ increased exponentially with time (Fig. 3): after 45 min of continuous tracer uptake, virtually every cell profile exhibited at least one AV⁺ profile. At that time, 30.0% (±2.4%, SEM of five measurements) of the endocytosed gold was present in AV. Therefore, although the earliest appearance of the tracer in AV was observed already after 10 min, the average time to reach AV is much longer. Considering the continuous uptake and assuming that the major part of endocytosed gold ends up in lysosomes, we estimated the average arrival time at ~30 min after internalization. Tracer uptake per se did not seem to induce AV formation or alter the composition of the AV population: the profile number of AV⁺ per cell profile (6.14 ± 0.57 [SEM]) or the proportion of AV⁺ (~30%; see Table I) did not change with time of endocytosis.

AV⁺ profiles exhibited, on average, six tracer particles, which were often not randomly dispersed throughout the AVi⁺, but rather appeared as small peripheral clusters, probably still at the site of delivery. The endocytosed gold was only rarely found inside small vesicles contained in the AVi. In such AVi, gold-containing vesicles had probably been engulfed by the AVi together with sequestered cytoplasm. In the majority of AV⁺, gold particles occurred not in internal vesicles, indicating that they usually enter AV by vesicular fusion. Within the first 20 min of uptake, >60% of the tracers was found to reside in AVi (Fig. 4). However, this dominance occurred only transiently. With time, gradually more tracer was found in AVd. The number of AV⁺ profiles, as indicated in the bars of Fig. 4, exhibited a parallel trend. The acquisition of tracers by the AV is not solely due to fusions with endosomes but is also due to mutual fusion events of AV. To investigate the delivery of tracer to AV by endosomes in more detail, we performed an analysis on fusion profiles between AV and endosomes that contained endocytosed gold (FP⁺).

**Fusion Profile Analysis**

The fusion profile approach was feasible in the present study because of the improved structural integrity with the modified cryosectioning technique. Occasionally, we observed a gold-carrying endocytic vesicle and an AV in what might be the initial phase of fusion, exhibiting a V-shaped membrane apposition (Fig. 5 b, arrowhead). The AV surface displayed a protrusion, and the endosome showed a corresponding indentation. The AV-endosome fusion profiles depicted in Figs. 2, b and c, and 5, b and c, respectively, may represent successive stages through which the fusion partners become confluent. Several fusion combinations were noticed. Of the 112 FP⁺ recorded, the tracer-bearing fusion partner for AV involved: (a) multivesicular endosomes (MVE, 64%), characterized by internal vesicles and a diameter of 200–500 nm (Figs. 2 c and 5 c), and (b) vesicular endosomes (VE, 36%), including small vesicles of the size of the earliest endocytic vesicles (Figs. 5 a and 7 a) and intermediate-sized electron lucent endosomes (100–250 nm in diameter), whose internalized gold tracers are affixed to the rim (Fig. 2 b). At all time points examined, both MVE and VE were seen to be involved in fusion (Fig. 6). In an attempt to further identify the endosomal elements involved in FP⁺, we screened around 20 fusion profiles in sections labeled for several endosomal and lysosomal markers, such as cathepsin D, MPR, and LGP-120. None of the markers used was abundant in the fusing MVE or VE, which made reliable quantitation cumbersome. However, the early endosomal marker, ASGP-R, labeled more prominently, occurring in ~65% of the fusion endosomes (Fig. 7 a). ASGP-R-positive MVE as well as VE were encountered (Fig. 7 a). ASGP-R was most abundant at the plasma membrane and in adjacent early endosomal elements (Geuze et al., 1983: Fig. 7 b). In this area, MVE- and VE-like structures containing endocytosed gold (Fig. 7 b) sometimes seemed to be involved in lateral sorting of the ASGP-R (Fig. 7 c) as described before (Geuze et al., 1983). Cathepsin D (Fig. 2 a) could not be detected in the endosomal part of FP⁺, but 40–45% of them were positive for LGP-120. In contrast to ASGP-R, LGP-120 is almost absent at the cell surface (Geuze et al., 1988). These observations are concomitant with the idea that the endosomal partners do undergo some maturation before their fusion with AV.

When FP⁺ were categorized according to the AV types, AVi, AVi/d, and AVd all were seen to be involved in fusion (Fig. 8). This indicates multiple entrance sites for endocytosed material. A further analysis of fusion frequencies did not show any apparent change in the types of AV and endosomes involved in fusions with time (Figs. 6 and 8) as opposed to the foregoing results of AV⁺ profile/tracer accumulation analysis (Fig. 4). For each time point, an average of ~60, 20, and 20% of FP⁺ involved AVi, AVi/d, and AVd, respectively (Table I, row 1). By far, the majority of FP⁺ occurred with AVi, suggesting that AVi represent the preferred site of tracer entry. To investigate whether this apparent fusion preference of AVi is merely due to the fact that there were more AVi than AVi/d and AVd available for fusion, we determined the relative number, volume, and surface densities of the AV types by morphometric analysis. The relative numerical, volume, and surface densities of AVi, AVi/d, and AVd were approxi-
Figure 2. Labeling patterns of cathepsin D and CAIII in MU picked-up cryosections. (a) Cells treated as in Fig. 1 b, but the section was immunolabeled for cathepsin D, which is not detectable in the AVi, moderately present in the AVi/d, and abundant in AVd. Cells in b and c had endocytosed 5-nm gold tracer for 20 and 40 min, respectively, before fixation and CAIII labeling. (b) Complex fusion profile of a CAIII-positive AVi, an AVd which is without CAIII labeling, and a VE that contains an endocytosed gold probe (arrow). Close to the AVd is another tracer-bearing VE. (c) Fusion profile of a tracer-bearing MVE and an AVi/d that exhibits a single limiting membrane and a CAIII-labeling concentration similar to that of the cytoplasm. Bars, 200 nm.
mately the same, i.e., around 35, 10, and 55%, respectively (Table I, rows 2, 3, and 4). By normalizing the fusion frequencies according to these values, we found a fusion preference of endocytic vesicles equal for AVi and AVi/d, but five to six times less for AVd (Table I, row 5). This clearly indicates that the preference of early AV as fusion partners for endocytic vesicles does not result from random encountering events, but rather may be governed by some specific recognition mechanism.

As to the frequency of FP\(^{1}\) in the sections, at the 40-min time point we screened 200 cell profiles and encountered 90 AV\(^{1}\) (Fig. 4) in a total of \(\approx 1,200\) AV. Among these were 12 FP\(^{1}\). About the same number of FP lacking tracer was found at this time. Thus, the fusion frequency may be as much as one per 50 AV profiles observed. Considering the sampling limitations of the 50-nm thick sections, we took this as an indication that endosome–AV fusions are common events.

**Discussion**

The main achievements of our present study are a more detailed definition of AV stages than was possible before and the identification of the sites where the endocytic and autophagic pathways merge. Both contributions relied on a better morphological resolution in cryosections processed according to a recently improved technique (Liou et al., 1996).

**Improved AV Morphology**

In previous studies on autophagy, we took advantage of the differential resistance to proteolysis of two cytosolic enzymes, SOD and CAIII, to determine the sequence of AV formation in hepatocytes, using quantitative immuno-EM. Those AV with a SOD/CAIII-labeling ratio similar to the cytosol were classified as AVi, those with a relatively high ratio, because of low CAIII and concentrated SOD, were termed AVd, while AVi/d took an intermediate position in CAIII/SOD ratio (Rabouille et al., 1993). Thus, CAIII rapidly disappears and SOD gradually accumulates in the maturation sequence AVi, AVi/d, and AVd. However, as in most cytochemical studies in plastic sections (Dunn 1990\(^{a}\); Yokota et al., 1995), the morphology of the AV in the cryosections was unsatisfactory (see also Tooze et al., 1990; Liou et al., 1993; Fengsrud et al., 1995), which hampered further investigations on the possible interactions of the autophagic and endocytic degradative pathways. In previous studies, we used the so-called sucrose pick-up method (Tokuyasu, 1973), in which dry cryosections are retrieved from the cryotome knife by letting them adhere to a drop of nearly saturated sucrose. A systematic search for the factors that caused the structural damage as observed in AV indicated that the high surface tension of the sucrose induced most of the damage. This could be circumvented by adding surface tension–lowering (methyl cellulose) and/or lipid-stabilizing (uranyl acetate) compounds to the cryosection retrieval solution. A significantly improved preservation of vulnerable cell structures, like AV, Golgi complexes, and endosomes was achieved by that modified method (Liou et al., 1996).

With this improved technique, the periphery of AV is left intact, revealing the multimembranous envelope of AVi. In addition, we found that the empty cleft between the sequestering membrane of AVi and its content, as shown in most studies so far, contains a lipid-like material (Fig. 1b; Liou et al., 1996). This may relate to observations on the enrichment of unsaturated fatty acids (Reunanen et al., 1985) and the deprivation of intramembrane particles in the wall of the AV (Punnonen et al., 1989). Most likely this material is derived from partial degradation of the multiple membranes or other membranes engulfed by the AV and suggests delivery of hydrolytic enzymes to AV...
as early as the AVi stage. This would be in accordance with our finding that AVi are capable of fusing with endosomes and lysosomes. The improved AV morphology allowed us to distinguish AVi, AVi/d, and AVd on morphological criteria. The immunolabeling characteristics for CAIII, SOD, and cathepsin D, as well as the SOD concentration ratios, were as described before (Rabouille et al., 1993).

**Convergence of Autophagic and Endocytic Pathways**

The notion that the autophagic and endocytic/degradative pathways interconnect stems from the work of Høyvik and colleagues (1987) and has been accepted widely. However, uncertainty remained on the precise site(s) where the pathways come together. Biochemical evidence for fusion of AV with the endosomal route at prelyosomal stage (Gordon and Seglen, 1988) found only limited support from morphological observations, which mostly indicated that late, degradative stages of autophagic structures are the most upstream sites in the autophagic route where the endocytotic route merges (Dunn, 1990b; Lawrence and Brown, 1992; Yokota et al., 1995).

**Figure 5.** Fusion profiles. Cells exposed to 5-nm gold tracers for 20 (a and c) and 25 min (b) before fixation. Sections were picked up by MU and air-dried (c) or underwent immunolabeling with anti-SOD antibodies (a and b). (a) Fusion between a small VE (arrow) and an AVi. A cluster of two gold particles (arrowhead) is also seen at another location. (b) Fusion between an electron lucent MVE (arrow) and an AVi. (c) Fusion between an MVE (arrow) and an AVd. Bars, 200 nm.
With the new cryosectioning technique, fusion profiles between autophagic and endocytic compartments, although not numerous, could easily be identified. Membrane continuities between tracer-containing endosomes and AV were considered to reflect fusion events. Two lines of evidence indicated that endocytosed gold tracer is delivered primarily to early AV. First, at early time points after the onset of endocytosis, <10% of tracer particles encountered in AV occurred in AVd. This increased to around 50% within the 45-min period that we studied. The particles contained in the AVi were often clustered at their periphery, reminiscent of delivery there. Second, when actual fusion profiles of AV and endocytic vesicles showing tracer (FP\(^+\)) were quantitatively analyzed over 45 min of continuous uptake, FP\(^+\) including AVi prevailed with 60% of all FP\(^+\). The frequency of FP\(^+\) with either AV type did not change over time and averaged at ~75% for AVi and AVi/d together. Only a minority of the FP\(^+\) occurred with AVd. Thus, all AV stages are capable of taking up endocytosed tracer. Morphometric measurements of volume and surface densities of AV types further indicated that AVi and AVi/d have at least a fivefold greater chance to fuse with endosomes than AVd. Usually we observed different AV types mixed together in the same cell region. Therefore, the fivefold preference is probably not due to some

![Image](Figure 6)

**Figure 6.** Endosome types involved in FP\(^+\). The FP\(^+\) were classified according to MVE and VE involved. The actual numbers of FP\(^+\) analyzed are given above the bars.

![Image](Figure 7)

**Figure 7.** ASGP-R labeling in FP\(^+\) and endosomes. Cells exposed for 40 min to 5-nm gold tracer. Section immunolabeled for ASGP-R with 10-nm gold. (a) Low ASGP-R labeling in tracer-carrying endocytic vesicles (arrowheads), adjacent to (top) or fusing with (bottom) an AVi/d. (b) ASGP-R labeling at the plasma membrane and in endosomal elements near the cell surface (ex, extra cellular space). Vesicles of the MVE (asterisk) and VE (arrowhead) type, containing endocytosed gold, are present in the same area. (c) As b. A VE-like vesicle with endocytosed gold and low ASGP-R labeling (arrowhead) is shown connected to an ASGP–R rich element (arrow), possibly a recycling endosome. Bars, 200 nm.

![Image](Figure 8)

**Figure 8.** AV types involved in FP\(^+\). The same FP\(^+\) data as used in Fig. 6 were classified according to the AV types.
topographical advantage of the earliest AV stages for meeting endosomes, but rather suggests specific transport and recognition mechanisms.

Together, our data show that the autophagic and endocytic pathways merge in a multipotential fashion with a main role for newly formed AV to fuse with endocytic structures. This is the first time that AVi have been shown to have this capacity. Even in a similar liver cell system, with BSA–gold complex as tracer, Lawrence and Brown (1992) did not find AVi as fusion partners for endosomes/lysosomes. Since AVi were similarly defined as multiple membrane bound, we reason that this inconsistency may be due to differences in the extent of profile search and/or their use of leupeptin, a cysteine proteinase inhibitor. The only other study concentrating on FP to our knowledge is that of Yokota et al. (1995), a study on liver cells in which the proliferation and autophagy of excess peroxisomes was enhanced by in vivo drug application. Fusion of endocytosed HRP-positive endosomes with peroxisome-containing AV was analyzed, both morphologically and in cell fractions. In contrast to our results, the authors found a clear preference of endosomes and lysosomes to fuse with late AV (92%). However, the morphological characterization of AV types was probably hampered by the enzyme cytochemical procedure and lack of sufficient morphological resolution. Together with prolonged drug treatment, this might have caused the apparent discrepancy.

In the present study, a solid systematic analysis of the endocytic compartments involved in fusion with AV was not feasible for two reasons. First, the endosomal system does not show a conspicuous morphological distinction between the various subcompartments. Second, we lack a reliable marker system, like SOD/CAII for the autophagic route, allowing definition of endocytic subcompartments. The endosomal partners involved in FP+ were lucent VE of variable size and MVE. The relative contribution of these endosome types to the FP+ did not change with time of gold uptake. We observed FP+ already after 10 min of endocytosis, indicating that early stages of endosomes can fuse with AV. This is supported by our finding that ASGP-R–positive endosomal vesicles were involved in FP+. The earliest timepoint of convergence between endosomes and AV reported so far was 30 min after uptake of HRP (Tooze et al., 1990; Punnonen et al., 1993). This discrepancy may be due to differences in the cell types studied or to insufficient morphology of autophagic compartments and FP in these earlier studies.

We thank René Scriwaneck, Maurits Niekert, and Tom van Rijn for excellent photographic assistance. Agnes Lee for correcting the manuscript, and Drs. Monica Fensgrud and Per Seglen (Oslo), Fred Meyer (Amsterdam), Catharine Rabouille, and Willem Stoorvogel (Utrecht) for constructive advice. The present work was made possible by a three-year research fellowship from the National Science Council, Republic of China in Taiwan, and a study grant from the Chang Gung College of Medicine and Technology to W. Liou.

Received for publication 28 May 1996 and in revised form 10 October 1996.

References

Aristila, A.U., and B.F. Trump. 1968. Studies on cellular autophagocytosis. The formation of autophagic vacuoles in the liver after glucagon administration. Am. J. Pathol. 53:687–733.

Dunn, W.A., Jr. 1990a. Studies on the mechanism of autophagy: formation of the autophagic vacuole. J. Cell Biol. 110:1923–1933.

Dunn, W.A., Jr. 1990b. Studies on the mechanism of autophagy: maturation of the autophagic vacuole. J. Cell Biol. 110:1935–1946.

Dunn, W.A., Jr. 1994. Autophagy and related mechanisms of lysosome-mediated protein degradation. Trends Cell Biol. 4:139–143.

Fengsrud, M., N. Ross, T. Berg, W. Liou, J.W. Slot, and P.O. Seglen. 1995. Ultrastructural and immunocytochemical characterization of autophagic vacuoles in isolated hepatocytes: effects of vinblastine and aspirin on vacuole distributions. Exp. Cell Res. 221:504–519.

Geelen, M.J.H., A.C. Beynen, R.Z. Christiansen, M.J. Lepreau-Jose, and D.M. Gibson. 1978. Short-term effects of insulin and glucagon on lipid synthesis in isolated rat hepatocytes. FEBS Lett. 95:320–330.

Geuze, H.J., J.W. Slot, G.J.A.M. Strous, H.F. Lodish, and A.L. Schwartz. 1983. Intracellular site of asialoglycoprotein receptor-ligand uncoupling: double-label immunoelectron microscopy during receptor-mediated endocytosis. Cell 32:277–287.

Geuze, H.J., W. Stoorvogel, G.J. Strous, J.W. Slot, J.E. Bleekemolen, and I. Mollman. 1988. Sorting of mannose 6-phosphate receptors and lysosomal membrane proteins in endocytic vesicles. J. Cell Biol. 107:2491–2501.

Gordon, P.B., and P.O. Seglen. 1988. Prelysosomal convergence of autophagy and endocytic pathways. Biophys. Res. Commun. 151:40–47.

Hayskja, H., P.B. Gordon, and P.O. Seglen. 1987. Convergence of autophagic and endocytic pathways at the level of the lysosome. Biochem. Soc. Trans. 15:964–965.

Lawrence, B.P., and W.J. Brown. 1992. Autophagic vacuoles rapidly fuse with preexisting lysosomes in cultured hepatocytes. J. Cell Sci. 102:515–526.

Liou, W., Y.Y. Chang, H.J. Geuze, G.J. Strous, J.D. Crapo, and J.W. Slot, 1993. Distribution of Cu Zn superoxide dismutase in rat liver. Free Radical Biol. Med. 14:201–207.

Liou, W., H.J. Geuze, and J.W. Slot. 1996. Improving structural integrity of cryosections for immunogold labeling. Histochem. Cell Biol. 106:41–58.

Punnonen, E.-L., K. Pihakaski, K. Mattila, K. Lounatmaa, and P. Hirsimäki. 1989. Intramembrane particles and filipin labeling on the membranes of autophagic vacuoles and lysosomes in mouse liver. Cell Tissue Res. 258:269–276.

Punnonen, E.-L., S. Autio, V.S. Marjomäki, and H. Reunanen. 1992. Autophagy, cathepsin L transport, and acidification in cultured rat fibroblasts. J. Histochem. Cytochem. 40:1579–1587.

Punnonen, E.-L., S. Autio, H. Kajja, and H. Reunanen. 1993. Autophagic vacuoles fuse with the prelysosomal compartment in cultured rat fibroblasts. Eur. J. Cell Biol. 61:54–66.

Rabouille, C., G.J. Strous, J.D. Crapo, H.J. Geuze, and J.W. Slot, 1993. The differential degradation of two cytosolic proteins as a tool to monitor autophagy in hepatocytes by immunocytochemistry. J. Cell Biol. 120:897–908.

Reunanen, H., E.-L. Punnonen, and P. Hirsimäki. 1985. Studies on vinblastine-induced autophagocytosis in mouse liver. V. A cytochemical study on the origin of membranes. Histochemistry. 83:513–517.

Roth, J., M. Bendayan, and L. Orcz. 1978. Ultrastructural localization of intra-cellular antigens by the use of protein A–gold complex. J. Histochem. Cytochem. 26:1074–1081.

Seglen, P.O. 1976. Preparation of isolated rat liver cells. Methods Cell Biol. 13: 79–88.

Seglen, P.O. 1987. Regulation of autophagic protein degradation in isolated liver cells. In Lysosomes: Their Role in Protein Breakdown. H. Glaumann and F.J. Ballard, editors. Academic Press, London. 369–414.

Slot, J.W., and H.J. Geuze. 1985. A new method of preparing gold probes for multiple-labeling cytochemistry. Eur. J. Cell Biol. 38:87–93.

Small, J.V., and J.E. Celis. 1978. Filmament arrangements in negatively stained tissues. J. Cell Biol. 77:308–325.

Stornhau, P.E., and P.O. Seglen. 1993. Evidence for acidity of prelysosomal autophagic/endocytic vacuoles (amphisomes). Biochem. J. 291:115–121.

Tokuyasu, K.T. 1973. A technique for ultracytology of cell suspensions and tissues. J. Cell Biol. 57:551–565.

Tooze, J., M. Hollinshead, T. Ludvig, K. Howell, B. Holfack, and H. Kern, 1990. In exocrine pancreas, the basolateral endocytic pathway converges with the autophagic pathway immediately after the early endosome. J. Cell Biol. 111:329–345.

Weibel, E.R. 1969. Stereological principles for morphometry in electron microscopic cytology. Int. Rev. Cytol. 26:235–302.

Yokota, S., M. Himeno, and K. Kato. 1995. Formation of autophagosomes during degradation of excess peroxisomes induced by dibutyryl cAMP. J. Histochem. Cytochem. 43:1549–1555.