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Eeva-Liisa Punnonen

Ultrastructural Studies on Cellular Autophagy

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Structure of Limiting Membranes and Route of Enzyme Delivery

Academic Dissertation

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ABSTRACT

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In autophagy, a portion of cytoplasm is first segregated into a membrane-bound vacuole which then acquires lysosomal enzymes. The present study investigated: 1) the structure of autophagic-vacuole membranes in Ehrlich ascites cells and mouse hepatocytes; 2) the role of microtubules in autophagy in Ehrlich ascites cells; and 3) the route of enzyme delivery to autophagic vacuoles in cultured rat fibroblasts.

The density of membrane particles in freeze-fracture replicas reflects the protein content of membranes. In hepatocytes, the membranes of early vacuoles were devoid of particles, whereas those in ascites cells contained particles. In both cell types, however, particle density was higher in later vacuoles. The membranes of nascent vacuoles were weakly labelled with filipin, which detects cholesterol, whereas those of the later vacuoles were heavily labelled. The increase in protein and cholesterol content may be associated with enzyme delivery, since the lysosomal membranes were rich in protein and cholesterol. The microtubule inhibitor vinblastine accumulated later autophagic vacuoles in Ehrlich ascites cells. Thus, microtubule disruption did not prevent the entry of lysosomal enzymes into nascent vacuoles. Since the rate of proteolysis did not increase, the accumulation must have been caused by retarded degradation in later vacuoles.

In fibroblasts, mannose 6-phosphate receptor (MPR), which serves as a receptor for lysosomal enzymes, and cathepsin L, a lysosomal proteinase, were detected in early autophagic vacuoles. Inhibitors of MPR-mediated transport did not prevent cathepsin L delivery. Thus, the enzyme is not transported directly from the trans-Golgi. On the other hand, studies with endocytic markers showed that autophagic vacuoles fused with late endosomes. Acidification of the vacuoles occurred concomitantly with cathepsin L delivery, but the inhibition of acidification did not prevent the transport of cathepsin L. This suggests that in fibroblasts late endosomes are an important source of lysosomal enzymes and perhaps also proton pumps for autophagic vacuoles.

Key words: Autophagy; endocytosis; electron microscopy; cytochemistry; immunocytochemistry.

List of original publications

This thesis is based on the following original publications, which will be referred to by their Roman numerals:

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

III. Punnonen. E.-L. & Reunanen, H. 1989: Effects of vinblastine, leucine and histidine, and 3-methyladenine on autophagy in Ehrlich ascites cells. - Experimental and Molecular Pathology 52: 87-97.

IV. Punnonen, E.-L., Autio, S., Marjomäki, V. S. & Reunanen, H. 1992: Autophagy, cathepsin L transport, and acidification in cultured rat fibroblasts. - The Journal of Histochemistry and Cytochemistry 40: 1579-1587.

V. Punnonen, E.-L., Autio, S., Kaija, H. & Reunanen, H. 1992: Autophagic vacuoles fuse with the prelysosomal compartment in cultured rat fibroblasts. - Manuscript.

Abbreviations

ADP	adenosine diphosphate
AMP	adenosine monophosphate
Arg	arginine
ATP	adenosine triphosphate
BSA	bovine serum albumin
CF	cationized ferritin
DAMP	3-(2,4-dinitroanilino)-3'-amino-N-methyl-dipropylamine
DMEM	Dulbecco's modification of Eagle's medium
ER	endoplasmic reticulum
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GDP	guanosine diphosphate
Gln	glutamine
Glu	glutamic acid
GTP	guanosine triphosphate
His	histidine
HRP	horseradish peroxidase
Leu	leucine
Lys	lysine
MPR	cation-independent mannose 6-phosphate receptor
NSF	N-ethylmaleimide sensitive factor
PBS	phosphate-buffered saline
Phe	phenylalanine
PLC	the prelysosomal compartment
RER	rough endoplasmic reticulum
TGN	trans-Golgi network

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1 INTRODUCTION

Subcellular components are degraded and resynthesized, "turned over", many times during the life span of a cell. The degradation is a central mechanism in the regulation of the cellular metabolism. Two mechanisms take care of the turnover, the lysosomal and the non-lysosomal pathways (Knowles & Ballard 1976, Hershko & Ciechanover 1982, Ciechanover 1987). Autophagy is the major pathway for the lysosomal degradation of intracellular components. Non-lysosomal degradation occurs either in the cytoplasm through the selective ubiquitin-mediated pathway (Finley & Varshavsky 1985, Hershko & Ciechanover 1992) or in the endoplasmic reticulum (Klausner & Sitia 1990). These pathways degrade abnormal and short-lived proteins and newly-synthesized incorrectly folded or assembled proteins, respectively. The main part of intracellular proteolysis occurs in lysosomes: more than 90% of long-lived proteins and a large proportion of short-lived proteins are degraded via autophagy (Bohley & Seglen 1992). Autophagic proteolysis is a non-selective process: to a certain extent any intracellular protein can be degraded in autophagic vacuoles. Autophagy participates in the regulation of cellular homeostasis: it increases under starvation conditions and during cessation of growth (Hendil 1981, Seglen & Bohley 1992).

2 REVIEW OF LITERATURE

2.1 The lysosomal system in mammalian cells

Almost every cell has a digestive compartment known as the lysosomal system (reviewed by de Duve & Wattiaux 1966, de Duve 1983, Pfeifer 1987). It degrades both intracellular and extracellular material in membrane-bound acidic vacuoles by means of lysosomal hydrolytic enzymes. The first step in the digestion is the engulfment or *sequestration* (or *segregation*) of the material destined to be degraded into a membrane-bound closed vacuole. If the sequestrated material is intracellular, the process is called *autophagy*. Engulfment of extracellular material is called *endocytosis* (engulfment of soluble material) or *phagocytosis* (engulfment of particles). Figure 1 summarizes the stages of sequestration in lysosomal degradation. Next, the vacuole becomes acidic and acquires hydrolytic enzymes, by fusing with vesicles belonging to the lysosomal system, and degradation begins. Degradation products either diffuse, or are transported, through the lysosomal membrane into the cytoplasm where they are used for the needs of the cell (Pisoni & Thoene 1991). The degradation of the segregated material sometimes remains incomplete, and partially degraded material accumulates inside the lysosomal vesicle. In this case, the vesicle becomes a residual body. Some cells are able to discharge the contents of residual bodies from the cell (Marzella & Glaumann 1980a, Tang et al. 1988), whereas in other cell types residual bodies accumulate in the cytoplasm and form lipofuscin pigment (Young 1982).

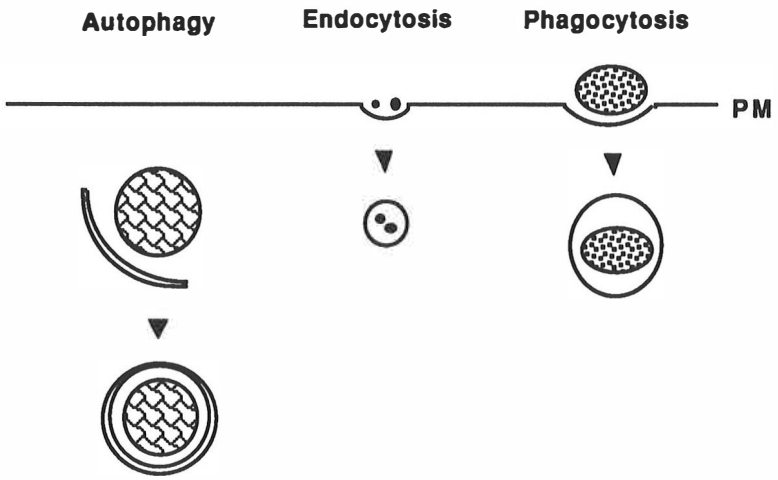

FIGURE 1. The sequestration stages in autophagocytosis, endosytosis and phagocytosis. Endosomes and phagosomes form from the plasma membrane (PM), whereas autophagosomes are formed by an intracellular membrane.

Lysosomal enzymes are able to degrade proteins, lipids, carbohydrates and nucleic acids (Tappel 1969). The pH-optimum of most lysosomal enzymes is 4-5 (Tappel 1969, Bohley & Seglen 1992), which corresponds to the intralysosomal pH (Ohkuma & Poole 1978, Tycko & Maxfield 1982). Lysosomal proteolysis is usually initiated by endopeptidases, which are rate-limiting, and continued by exopeptidases. Cathepsins D and L are the main lysosomal endopeptidases (Bohley & Seglen 1992). Cathepsin L is probably obligatory for the initiation of lysosomal proteolysis (Furuno et al. 1985, Kopitz et al. 1990, Kominami et al. 1991). The concentration of proteases in lysosomes is high, which explains the rapid degradation (half-life less than 10 min) of proteins in lysosomes. In contrast to this, the half-life of lysosomal enzymes is very long, lasting from a few days to several weeks (Bohley & Seglen 1992).

All lysosomal enzymes are glycoproteins. The transport of soluble enzymes to lysosomes is mediated by a mannose 6-phosphate recognition marker (Hickman & Neufeld 1972, Pfeffer 1991), whereas membrane-associated enzymes are transported by a mannose 6-phosphate –independent manner (Tsuji et al. 1988, Waheed et al. 1988, Ginsel & Fransen 1991, Rijnboutt et al. 1991). Cathepsins B and L, and acid phosphatase are examples of soluble and membrane-associated lysosomal enzymes, respectively. The polypeptide chains of the enzymes are synthesized on membrane-bound ribosomes of the rough endoplasmic reticulum (RER) and translocated into the lumen of the RER (reviewed by von Figura & Hasilik 1986, Conner et al. 1987, Kornfeld & Mellman 1989, Hasilik 1992). In the RER lumen, the polypeptide chains are glycosylated, and, in case

of soluble enzymes, mannose 6-phosphate residues are linked to them. A preformed oligosaccharide containing glucose, mannose and N-acetylglucosamine is first linked to selected asparagine residues. The oligosaccharide is then modified by removal of glucose and mannose residues. The synthesis of mannose 6-phosphate occurs after transport to the cis-Golgi. First, N-acetylglucosamine 1-phosphate is transferred to one or more mannose residues. Then, N-acetylglucosamine residue is removed to generate mannose 6-phosphate. The enzyme precursors are then transported to the trans-Golgi, where the molecules are recognized by specific mannose 6-phosphate receptors (reviewed by von Figura & Hasilik 1986, Goldberg 1987, Kornfeld & Mellman 1989, Hasilik 1992).

Two different receptors for mannose 6-phosphate have been characterized. One has a molecular weight of 215 kDa (Sahagian et al. 1981, Goldberg et al. 1983), and the other of 46 kDa (Hoflack & Kornfeld 1985). The larger receptor (MPR) is known to transport newly synthesized enzymes, by means of small clathrin-coated vesicles which bud from the trans-Golgi network (TGN), into a prelysosomal compartment (PLC) (Griffiths et al. 1988). As the pH in the PLC is below 6, the enzymes dissociate from the MPRs and the receptors are recycled back to the TGN (Duncan & Kornfeld 1988, Goda & Pfeffer 1988). A small proportion of lysosomal enzymes is secreted under normal conditions (Neufeld et al. 1975, Creek & Sly 1984). MPR also mediates the endocytosis of exogenous lysosomal enzymes by recycling between the plasma membrane and the PLC (von Figura & Hasilik 1986, Kornfeld & Mellman 1989, Pfeffer 1991). The functions of the smaller mannose 6-phosphate receptor are less well known, but it has been suggested to mediate the secretion of lysosomal enzymes (Chao et al. 1990). Lysosomes presumably bud off or mature from the PLC (Griffiths 1989). The PLC contains significant amounts of MPR and lysosomal enzymes (Griffiths et al. 1988). Furthermore, the PLC accumulates endocytic markers (Griffiths et al. 1988, 1990, Griffiths 1989). Mature lysosomes, in contrast, do not contain MPR (Sahagian & Neufeld 1983, Geuze et al. 1984a, von Figura et al. 1984). After transport to the PLC and lysosomes, most lysosomal enzymes are proteolytically processed. Proteolytic processing usually activates the enzymatically inactive precursor molecules (Hasilik 1992).

Lysosomal membrane proteins and membrane-associated enzymes are targeted to lysosomes by a mannose 6-phosphate independent manner. The targeting signal has been located to the cytoplasmic tail of the proteins. Fusion of the cytoplasmic tail of a human lysosomal membrane glycoprotein, h-lamp-1, to a cell surface reporter glycoprotein caused transport of the chimeric protein to the lysosomal membrane (Williams & Fukuda 1990). Similarly, the cytoplasmic tail of acid phosphatase, when linked to hemagglutinin, a cell surface protein, was sufficient to target the chimera to lysosomes (Peters et al. 1990).

A selective signal-mediated uptake of proteins into lysosomes

from the cytoplasm has been described. In fibroblasts under serum deprivation, lysosomal degradation of certain microinjected proteins increased twofold (Dice et al. 1990). The increased degradation was found to be selective for proteins containing peptide sequences related to Lys-Phe-Glu-Arg-Gln. The proteins are transported into lysosomes, probably directly through the lysosomal membrane, by an unknown mechanism mediated by a 70 kDa heat shock protein (Chiang et al. 1989, Dice 1990).

The membranes bordering the the lysosomal organelles are resistant to hydrolysis by the lysosomal enzymes, as well as capable of the acidification of the organellar lumen and transportation of the degradation products out of the organelles (reviewed by Lloyd & Forster 1986, Forster & Lloyd 1988, Pisoni & Thoene 1991). Several glycoproteins of the lysosomal membrane have been characterized, and they are all heavily glycosylated and sialylated (Chen et al. 1985, Lewis et al. 1985, Granger et al. 1990). An ATP-driven proton pump, vacuolar-type H^+-ATPase (Moriyama & Nelson 1989, Yoshimori et al. 1991), and several amino acid transporters (Pisoni & Thoene 1991), have also been identified.

The classical lysosome concept (reviewed by de Duve & Wattiaux 1966) assumed that primary lysosomes bud from the trans-Golgi apparatus. As these primary lysosomes acquired degradable material from the autophagic or endocytic route, they were thought to mature into secondary lysosomes. The terms primary and secondary lysosome are not used in the new concept of the lysosomal system. Lysosomes are defined as MPR negative, hydrolase positive vesicles which form the terminal degradative compartment in the cells (Kornfeld & Mellman 1989). The current lysosomal concept is schematically presented in Figure 2.

2.2 Endocytosis

The endocytic pathway has been extensively studied (reviewed by Goldstein et al. 1985, Pastan & Willingham 1985, Gruenberg & Howell 1989, Smythe & Warren 1991). Several model systems have been characterized biochemically and morphologically, e.g. the endocytosis of low density lipoprotein and its receptor (Goldstein & Brown 1974, Anderson et al. 1982, Brown et al. 1983), adsorptive endocytosis of Semliki Forest virus (Marsh & Helenius 1980, Kielian et al. 1986), endocytosis of transferrin and its receptor (Bleil & Bretscher 1982, Ciechanover et al. 1983, Harding et al. 1983, Hopkins & Trowbridge 1983, Klausner et al. 1983), epidermal growth factor and its receptor (Dunn & Hubbard 1984), asialoglycoproteins and their receptor (Wall et al. 1980, Geuze et al. 1984b), lysosomal enzymes and MPR (Gabel & Foster 1986, Woods et al. 1989), and protein toxins (Sandvig et al. 1989). Cell-free assays have also

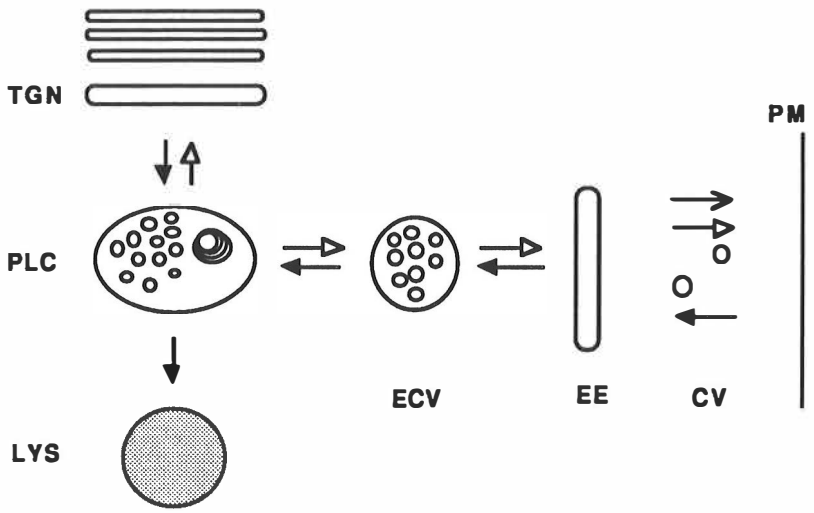

FIGURE 2. Schematic drawing of the lysosomal and endosomal systems in a non-polarized cell. Lysosomal enzymes are packaged in the trans-Golgi network (**TGN**) into clathrin-coated vesicles and transported into the prelysosomal compartment (**PLC**). The mannose 6-phosphate receptors recycle back to the TGN (blank arrow). Lysosomes (**LYS**) either mature from or bud off the PLC. In endocytosis, extracellular molecules enter the cytoplasm in clathrin-coated or uncoated vesicles (**CV**) which bud from the plasma membrane (**PM**). The vesicles transport endocytosed material into vesicular-tubular early endosomes (**EE**). Several receptors mediating endocytosis of various ligands recycle from early endosomes back to the plasma membrane (arrow). MPRs mediating endocytosis of lysosomal enzymes recycle to the plasma membrane from the PLC (blank arrows). The endocytosed material is transported in putative endocytic carrier vesicles (**ECV**) to the PLC, and from there, to lysosomes. Alternatively, early endosomes may mature into the PLC.

been developed for different stages in the endocytic pathway (Gruenberg & Howell 1986, Braell 1987, Goda & Pfeffer 1989, Mullock et al. 1989).

Receptor-mediated uptake of soluble molecules occurs via clathrin-coated pits, which bud from the plasma membrane and form coated vesicles. Coated vesicles lose their coat and then fuse with the early endosomal compartment located at the cell periphery. There is also evidence that non-coated vesicles may participate in the transfer of material to early endosomes (Tran et al. 1987, Hansen et al. 1991). In the mildly acidic early endosomes, many receptors dissociate from their ligands. The receptors are recycled back to the cell surface, and ligands transported to lysosomes for degradation. From early endosomes, the internalized material is transported to perinuclear late endosomes,

which correspond to the PLC (Griffiths et al. 1988, Schmid et al. 1988, Woods et al. 1989), and finally to mature lysosomes. The pH is 6.0-6.3 in early endosomes (Kielian et al. 1986, Sipe & Murphy 1987, Yamashiro & Maxfield 1987), and below 5.3 in late endosomes (Schmid et al. 1989).

Three models of the mechanism of endocytic transfer have been presented. According to the vesicular shuttling model, early and late endosomes are preexisting compartments of the cells, and materials are transported from early to late endosomes in shuttling vesicles (Gruenberg et al. 1989, Griffiths & Gruenberg 1991). The maturation model suggests that coated vesicles derived from the plasma membrane lose their coat and fuse together to form an early endosome. Early endosomes then mature into late endosomes and lysosomes by gradually receiving lysosomal proteins and releasing the recycling vesicles back to the plasma membrane (Murphy 1991, Stoorvogel et al. 1991, Dunn & Maxfield 1992). The third model suggests that internalized material moves along a continuous endosomal reticulum from the cell periphery to the perinuclear region (Hopkins et al. 1990). Transport between the plasma membrane and early endosomes does not require intact microtubules, whereas transport from early endosomes to the PLC does (Gruenberg & Howell 1989, Gruenberg et al. 1989). Along the endocytic pathway, the PLC is the first organelle with a significantly acidic pH and large amounts of lysosomal enzymes and lysosomal membrane proteins (Griffiths et al. 1988). The endocytic pathway is schematically presented in Figure 2.

2.3 Molecular mechanisms of vesicle-mediated transport

The original proposal of vesicle-mediated transport was made by Palade (1975). Small, short-lived vesicles bud from the donor compartment, are targeted to the acceptor compartment, and then rapidly fuse with its membrane. The molecular mechanisms of membrane traffic have been extensively studied during recent years using cell-free assays and yeast genetics (reviewed by Gruenberg & Clague 1992, Pryer et al. 1992, Schekman 1992). Vesicle-mediated transport is dependent on ATP and cytosolic proteins. A number of these proteins have been identified. These include small GTP-binding proteins, proteins forming the coats of transport vesicles, i.e. clathrin in the TGN and plasma membrane and COPs (coat proteins) in the Golgi apparatus (Waters et al. 1991), and proteins obligatory for the fusion of vesicles with the target membrane. N-ethylmaleimide-sensitive factor (NSF), and soluble NSF attachment proteins (SNAPs) are necessary for the fusion of intra-Golgi transport vesicles with the acceptor membrane (Orci et al. 1989, Clary et al. 1990). NSF

may also have a role in other stages of vesicular transport (Rothman & Orci 1992).

Small monomeric GTP-binding proteins (20-25 kDa) regulate both coated and uncoated vesicle-mediated transport (Rexach & Schekman 1991, Schekman 1992). Originally, the yeast gene *SEC4*, encoding a protein necessary for the targeting or fusion of secretory vesicles to the plasma membrane, was found to encode a small GTP-binding protein homologous to the *ras* proto-oncogene product (Salminen & Novick 1987, Bourne et al. 1990). Homologous genes were then detected in mammalian cells. The proteins, known as rab proteins, were originally cloned from a rat brain library (Touchot et al. 1987). In addition, ADP-ribosylation factor (ARF), a member of another small GTP-binding protein subfamily (Bourne et al. 1990), participates in vesicle transport in the Golgi apparatus (Stearns et al. 1990).

In mammalian cells, rab proteins have been localized to the cytoplasmic face of specific intracellular compartments (reviewed by Goud & McCaffrey 1991, Gruenberg & Clague 1992). The proteins are also found free in the cytoplasm. Rab1 and rab2 are located in the cis-Golgi, rab4 and rab5 in the plasma membrane and early endosomes, rab6 in the cis and medial Golgi, and rab7 in the PLC. The association of rab proteins with the specific organelles or compartments is mediated by a signal within the carboxyl-terminal hypervariable domain (Chavrier et al. 1991). The highly restricted intracellular distribution of rab proteins suggests that different proteins may act at each stage in the vesicular transport. Rab5 was found to be necessary for the homotypic fusion of early endosomes in a cell-free assay (Gorvel et al. 1991). It was also shown to regulate transport from the plasma membrane to early endosomes in cultured cells (Bucci et al. 1992). Further, mutant forms of rab1 and rab2 were found to inhibit transport from the ER to the Golgi apparatus (Tisdale et al. 1992).

Small GTP-binding proteins undergo a conformational switch when GDP is exchanged for GTP. The switch is reversed during GTP hydrolysis (Bourne et al. 1990). This can be used to introduce vectoriality to the transport processes via the regulation of nucleotide exchange and GTP hydrolysis. Possible regulators are proteins known to interact with rab proteins, e.g. guanine nucleotide releasing factor (GRF) and GTPase activating protein (GAP) (Bourne et al. 1990). In addition, protein phosphorylation may regulate the activity of rab proteins (Bailly et al. 1991). The actual mechanism by which small GTP-binding proteins participate in membrane transport is not understood but it has been proposed that they ensure the unidirectional delivery of vesicles to their targets. A model for the SEC4 function in yeast has been suggested (Walworth et al. 1989). According to this model, GDP bound to SEC4 is first exchanged for GTP. The GTP-bound conformation of SEC4 then recognizes an attachment site in a secretory vesicle. An effector protein on the plasma mem-

brane then recognizes the complex, and this leads to exocytosis. SEC4 is then released from the effector by a mechanism which requires GTP hydrolysis.

2.4 Autophagy

2.4.1 Mechanisms of autophagic sequestration

In the first stage of autophagy, a flattened membrane sack surrounds a portion of cytoplasm and forms a closed vacuole around it. The resulting vacuole is known as an *autophagosome*, and it is surrounded by two separate membranes (Arstila & Trump 1968, Ericsson 1969b). In the second stage, the autophagosome acquires lysosomal enzymes, by fusing with vesicles earlier assumed to be lysosomes (Arstila & Trump 1968, 1969, Ericsson 1969a, Deter 1971). After this stage, the vacuole is called an *autolysosome*. The term *autophagic vacuole* refers to an autophagosome or autolysosome.

In addition to autophagy or macroautophagy which was described above, two other types of autophagic sequestration have been observed: microautophagy and crinophagy. In *microautophagy*, lysosomes "endocytose" portions of cytoplasm by forming invaginations in their limiting membranes. These invaginations are internalized as intralysosomal vesicles (Ahlberg et al. 1982, Mortimore et al. 1983, de Waal et al. 1986). Alternatively, the whole lysosome may flatten and wrap itself around a portion of cytoplasm (Sakai & Ogawa 1982, Sakai et al. 1989a). In *crinophagy*, storage granules of the regulated secretory pathway fuse with lysosomes directly, without being sequestrated into macroautophagosomes (Glaumann et al. 1981, Poole et al. 1981, Ahlberg et al. 1987a). The term 'autophagy' is usually used to refer to macroautophagy. Figure 3 summarizes the three types of autophagic sequestration.

Macroautophagic segregation is a nonselective bulk process (Hopgood et al. 1988, Rogers & Rechsteiner 1988, Kopitz et al. 1990). This means that any cytoplasmic constituents can be degraded by autophagy at least to a certain extent (Seglen & Bohley 1992). Results arguing for a selective autophagic degradation (Lardeux & Mortimore 1987, Luiken et al. 1992) are most probably dealing with either non-lysosomal degradation, or the microautophagic pathway (reviewed by Seglen & Bohley 1992).

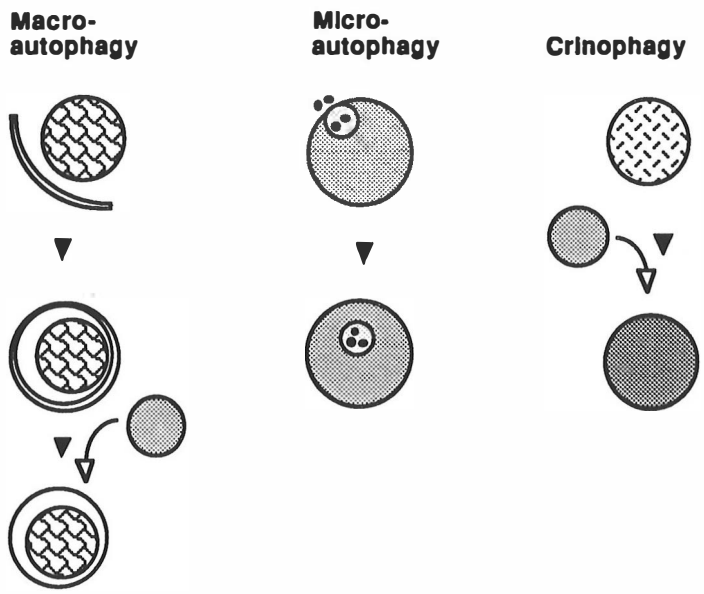

FIGURE 3. The mechanisms of autophagic sequestration. In macroautophagy, a portion of cytoplasm is first segregated into an autophagosome, a double-membrane bound vacuole devoid of lysosomal enzymes. This vacuole acquires enzymes by fusing with lysosomal vesicles which leads to degradation of the inner limiting membrane and the segregated cytoplasm. In microautophagy, lysosomes internalize small parts of the cytoplasm by a process equivalent to endocytosis. In crinophagy, secretory storage granules fuse directly with lysosomes.

2.4.2 Significance of autophagy

After the autophagic pathway of degradation had been discovered (reviewed by de Duve & Wattiaux 1966), it was first considered to be mainly associated with sublethal cell injury and cell death (reviewed by Ericsson 1969a, Lockshin & Beaulaton 1981), while having little physiological significance. Newer studies have, however, revealed that autophagy is the major pathway for the continuous turnover of cytosolic components and organelles, and it has significant roles in both cellular metabolic homeostasis and growth control (reviewed by Ericsson 1969a, Glaumann et al. 1981, Marzella et al. 1981, Hirsimäki et al. 1983, Henell & Glaumann 1984, Marzella & Glaumann 1987, Mortimore & Pösö 1987, Seglen & Bohley 1992).

Deprivation of nutrients increases the rate of protein degradation in cells. Increased autophagy has been shown to be the cause of this enhanced degradation (Mortimore & Ward 1981, Schworer et al. 1981,

Lee et al. 1989, Balavoine et al. 1990). Under nutrient deprivation, about 4%/h of cytoplasmic components are degraded by autophagy in isolated hepatocytes (Seglen & Bohley 1992). Moreover, autophagy is also involved in the basal protein degradation which occurs under normal nutritional conditions (Mortimore & Ward 1981, Furuno et al. 1982, Hutson & Mortimore 1982, Kominami et al. 1983, Henell & Glaumann 1984, 1985, Heydrick et al. 1991). Under these conditions, 1.34%/h of cytoplasm is segregated into autophagic vacuoles in hepatocytes (Henell & Glaumann 1985). Some studies have suggested that microautophagy may be the main autophagic pathway under basal conditions (Mortimore et al. 1983, de Waal et al. 1986, Mortimore & Pösö 1987).

The autophagic pathway degrades both short and long-lived proteins (Ahlberg et al. 1985, Henell et al. 1987). It has been estimated that autophagy accounts for 70% and 90% of the degradation of short and long-lived proteins, respectively (Marzella & Glaumann 1987, Bohley & Seglen 1992). The remaining 30% and 10% of protein degradation occurs in the cytoplasm and ER (Knowles & Ballard 1976, Klausner & Sitia 1990, Hershko & Ciechanover 1992). Direct evidence has also been presented to show that proteins of the ER, glucosidase II (Lucocq et al. 1986), cytochrome P450 and NADPH-cytochrome P450 reductase (Masaki et al. 1987), as well as a cytosolic protein, aspartate aminotransferase (Sato et al. 1988), are degraded in autolysosomes.

In most animal cell types and tissues, the major reason for increased amount of protein during growth is a decreased rate of protein degradation, as the rate of protein synthesis remains unchanged (Scornik & Botbol 1987). The slower rate of protein degradation has been shown to be due to decreased autophagy (Hendil 1981, Papadopoulos & Pfeifer 1987, Tessitore et al. 1987, 1988, Pfeifer et al. 1988, Tayek et al. 1988, Rosenwald 1990). In addition, the volume density of autophagic vacuoles has been found to be lower in several growing tissues as compared with corresponding non-growing tissues (Pfeifer 1979, Dämmrich & Pfeifer 1983, Bahro et al. 1987, Müller et al. 1987, Jurilj & Pfeifer 1990, de Almeida Barbosa et al. 1992, Han et al. 1992). In some types of muscular dystrophy, increased autophagic degradation in myocytes may be the cause of muscle degeneration (Kominami et al. 1987, Kalimo et al. 1988).

The regulation of growth rate and of autophagy is altered in transformed cells. In general, transformed cell lines posses a lower rate of protein degradation than their normal counterparts (Gunn et al. 1977, Knecht et al. 1984, Ahlberg et al. 1987b, Ballard 1987). In particular, autophagic degradation responds differently to amino-acid deprivation; only a slight increase occurs in transformed cells as compared with corresponding normal cells (Schwarze & Seglen 1985, Ballard 1987, Yucel et al. 1989). In vivo, reduced autophagic degradation may give the transformed cells an anabolic advantage and increase their relative growth rate (Schwarze & Seglen 1985, Ahlberg et al. 1987b).

2.4.3 Origin and structure of the limiting membranes of macroautophagic vacuoles

The origin of the membranes which form new autophagosomes has been the subject of long debate. Ashford and Porter (1962) and Pfeifer (1971) proposed that the membranes are synthesized de novo in the cytoplasm. However, most studies suggest that the segregating cisternae are derived from preexisting cytoplasmic membranes (reviewed by Hirsimäki et al. 1983, Marzella & Glaumann 1987). The membranes of autophagosomes have been studied using ultrastructural, cytochemical, and immunocytochemical methods, which are summarized below.

ER is the most studied candidate for autophagosome membranes. In ultrastructural studies, continuities of ER cisternae with the limiting membranes of autophagic vacuoles have been reported (Novikoff & Shin 1978), or membranes resembling cisternae of rough ER have been observed to form new autophagosomes (Marzella & Glaumann 1980a, Yu & Marzella 1988). Morphological studies have also revealed that the two membranes bordering nascent autophagosomes belong to the endoplasmic or thin membrane class (5-7 nm) (Arstila & Trump 1968, Ericsson 1969b). During the maturation of the autophagic vacuole, the inner membrane thickens; later, the outer membrane also thickens. Next, the inner membrane disappears, probably as a result of digestion by lysosomal enzymes (Arstila & Trump 1968, Marzella & Glaumann 1980a).

Cytochemical methods have been used to detect ER marker enzymes on the membranes of autophagosomes. Low levels of inosine diphosphatase and glucose 6-phosphatase activities were detected on the membranes of some double-membrane limited autophagic vacuoles in rat liver (Arstila & Trump 1968, Ericsson 1969b, Gray et al. 1981), but not in mouse liver (Hirsimäki & Reunanen 1980, Reunanen & Hirsimäki 1983) or rat Leydig cells (Tang et al. 1988). In mouse exocrine pancreas, some autophagic vacuoles showed weak lipase activity (Reunanen et al. 1988a). In these cells, lipase is a marker of the ER and Golgi apparatus.

Immunocytochemical studies on the origin of autophagosome membranes in rat liver have also been conducted. Dunn (1990a) reported that antibodies produced against RER integral membrane proteins (14, 25 and 40 kDa), ribophorin II, and secretory proteins (albumin and $alpha_{2\mu}$-globulin) labelled 35-79% of the membranes of nascent autophagic vacuoles. However, Furuno et al. (1990) did not observe carboxyesterase E1, a microsomal marker, or albumin, in these membranes. In addition, Yamamoto et al. (1990a) did not observe cytochrome P-450 on the autophagosome membranes. These conflicting results have been interpreted to suggest that autophagosomes are formed by modified, ribosome-free portions of ER (Ericsson 1969b, Dunn 1990a, Furuno et al. 1990).

Some studies have suggested that mature Golgi cisternae may form new autophagosomes (Frank & Christensen 1968, Jaeken & Thines-Sempoux 1981). In cell types which have a well developed Golgi apparatus, autophagic vacuoles have been observed to arise from acid-phosphatase positive cisternae presumably belonging to the Golgi-associated membrane reticulum, GERL (Novikoff et al. 1971, Decker 1974, Paavola 1978, Bouldin et al. 1981) which probably corresponds to the TGN.

The most recent model for the origin of membranes was presented by Seglen (1987). According to this model, autophagosomes are formed by a specific organelle called a phagophore. Seglen describes phagophores as "condensed, non-sequestering structures in the cytoplasm or at various stages of spreading, eventually forming the walls of the autophagosomes" (Seglen & Bohley 1992). So far, the phagophore has been characterized by morphological methods only (Seglen 1987). However, some cytochemical results by other groups can be interpreted to support this model. Lectin binding experiments have revealed that phagophore and autophagosome membranes contain complex oligosaccharides which can originate only from some post-Golgi source (Willemer et al. 1990, Yamamoto et al. 1990b). In addition, Yamamoto et al. (1990a) reported that antibodies produced against antigens of autophagic vacuoles labelled the phagophore and autophagosome membranes but not the ER membranes. Seglen interpreted the presence of ER markers in autophagosomes as an indication of a direct transport pathway from the ER to nascent autophagosomes (Seglen & Bohley 1992).

Osmium and other impregnation techniques have been used to detect lipid and carbohydrate components in the membranes of autophagic vacuoles. Unbuffered OsO_4 stains the cisternae at the forming face of the Golgi apparatus, and ER in some cells (reviewed by Hirsimäki et al. 1983). Unbuffered OsO_4 also heavily stains the cavity between the two membranes of nascent autophagic vacuoles (Arstila et al. 1972, McDowell 1974, Hirsimäki & Reunanen 1980, Reunanen et al. 1988a), but not the membranes of older vacuoles (Hirsimäki & Reunanen 1980, Reunanen et al. 1988a). A similar staining pattern of the membranes of autophagic vacuoles was observed using uranyl-lead-copper impregnation and imidazole-buffered OsO_4 staining (Reunanen et al. 1985, 1988a). The latter technique stains unsaturated fatty acids (Angermüller & Fahimi 1982). The inner surface of the autophagic-vacuole membrane also has more anionic sites, revealed by the binding of cationic ferritin, than the other cellular membranes (Sakai et al. 1989b). Using impregnation techniques which stain carbohydrates, Neiss (1986) showed that all single-membrane, and some double-membrane, limited autophagic vacuoles carry a coat of glycoconjugates on the inner surface of the limiting membrane. Lysosomal vesicles also carried this coat.

Freeze-fracture studies have revealed that, compared with other

cellular membranes, the membranes of double-membrane limited autophagic vacuoles contain only few intramembrane particles (Réz & Meldolesi 1980, Hirsimäki et al. 1982). Intramembrane particles are structures visible in freeze-fracture replicas, and they presumably correspond to membrane proteins (Rash et al. 1981). Furthermore, the limiting membranes of older, single-membrane limited vacuoles contain more particles than the early vacuoles, but still considerably less than other cellular membranes (Réz & Meldolesi 1980).

In summary, in spite of several studies, the origin of autophagosome membranes remains unknown. However, studies have revealed several structural characteristics of the membranes bordering autophagosomes and later autophagic vacuoles. Autophagosome membranes differ from the other cellular membranes in several cytochemical stainings. In addition, the composition of the membranes changes during the maturation of autophagic vacuoles.

2.4.4 Acquisition of lysosomal enzymes into autophagosomes

According to early ultrastructural and cytochemical studies, autophagosomes were suggested to acquire lysosomal enzymes by fusing with primary or secondary lysosomes (de Duve & Wattiaux 1966, Arstila & Trump 1968, 1969, Jaeken & Thines-Sempoux 1981). Biochemical and morphometric studies on subcellular fractions (Deter 1971) and intact cells (Ishikawa et al. 1983) as well as the labelling of secondary lysosomes with electron-dense markers (Ericsson 1969a, Deter 1975, Tang et al. 1988) proved that fusion between autophagosomes and endosomes or lysosomes occurred frequently. However, enzyme delivery has not yet been characterized according to the new lysosomal concept.

The stages in the delivery of lysosomal enzymes to autophagic vacuoles have been studied using acid-phosphatase cytochemistry and immunocytochemistry to localize several lysosomal hydrolases. The enzyme activity first appears between the two limiting membranes, presumably as a result of fusion between a lysosomal vesicle and the outer limiting membrane (Arstila & Trump 1968, Hirsimäki & Reunanen 1980, Dunn 1990b, Furuno et al. 1990). Next, enzyme activity appears in the matrix of the vacuoles (Arstila et al. 1972), probably due to digestion of the inner limiting membrane.

The average half-life of an autophagic vacuole is 6-9 min (Schworer et al. 1981, Kovács 1983, Papadopoulos & Pfeifer 1986, Kovács et al. 1987). The products of degradation cross the limiting membrane and enter the cytoplasm where they are used for energy production or the synthesis of new macromolecules (Gahl 1989). Proteins are degraded faster than lipids (Henell et al. 1983). Undegraded material may accumu-

late in autophagic vacuoles which then become residual bodies. The lysosomal degradation of phagocytosed mitochondria or microsomes in Kupffer cells leads to the accumulation of lipid-laden residual bodies (Glaumann et al. 1975a, b).

2.4.5 Energy requirements of autophagy

Autophagy has long been known to be energy-dependent (Shelburne et al. 1973, Sakai & Ogawa 1982). The energy requirements of the different stages in the autophagic pathway have been studied. Overall autophagic degradation was shown to be ATP-dependent (Plomp et al. 1987, 1989). Since the pH optimum of most lysosomal enzymes is acidic (Tappel 1969), the sequestrated cytoplasm must become acidic before the degradation begins. The energy requirements of autophagic degradation may be due to an ATP-dependent proton pump which acidifies the contents of the vacuoles (Schneider 1983, Ahlberg & Glaumann 1985, Ohkuma 1987, Plomp et al. 1987, Schellens & Meijer 1991).

In isolated hepatocytes, autophagic sequestration was shown to be inhibited by partial ATP depletion (Schellens et al. 1988, 1990). Interestingly, different results were reported for Ehrlich ascites cells. In these cells, partial ATP depletion led to an accumulation of acid phosphatase -negative autophagosomes, suggesting that the formation of new autophagosomes was not inhibited, whereas the delivery of lysosomal enzymes and the maturation of these vacuoles were (Reunanen & Nykänen 1988, Reunanen et al. 1991). All the stages in vesicle-mediated transport in yeast and animal cells appear to be ATP-dependent (reviewed by Pryer et al. 1992). It is probable, therefore, that the enzyme delivery stage in autophagy is also dependent on metabolic energy.

2.4.6 Physiological regulation of autophagy

The physiological regulation of autophagy is mainly exerted during the first stage, sequestration. Amino acids are the most prominent regulators (reviewed by Mortimore & Pösö 1987, Mortimore 1987, Seglen 1987). Deprivation of amino acids has been shown to induce (Mitchener et al. 1976, Mortimore & Schworer 1977), and a physiological mixture of amino acids, to inhibit (Seglen et al. 1980, Kovács et al. 1981) the formation of new autophagosomes. Leucine probably has a special role; it was found to be indispensable for the maximum inhibition of lysosomal protein degradation induced by the complete mixture of amino acids (Mortimore 1987, Seglen 1987, Caro et al. 1989). Furthermore, leucine

and histidine were found to be the most effective inhibitors of autophagic segregation in isolated hepatocytes (Seglen & Gordon 1984). Asparagine, instead, was found to inhibit the fusion between autophagosomes and lysosomes (Gordon & Seglen 1988, Høyvik et al. 1991).

The pancreatic hormones insulin and glucagon regulate autophagy in vivo. Insulin inhibits (Pfeifer 1978, Pfeifer & Warmuth-Metz 1983), and glucagon stimulates (Ashford & Porter 1962, Arstila & Trump 1968), the formation of new autophagic vacuoles. In cultured fibroblasts, deprivation of serum and growth factors increases the lysosomal degradation of cytosolic proteins (Auteri et al. 1983, Slot et al. 1986, Ballard 1987). In addition, adrenaline and other adrenergic agonists inhibit autophagic sequestration in isolated hepatocytes (Gordon et al. 1991). In vivo, circadian variation of autophagic degradation occurs in many tissues (Pfeifer & Strauss 1981, de Waal et al. 1986). The second messenger cyclic AMP and protein phosphorylation have been shown to regulate sequestration (Holen et al. 1991, 1992). Both cyclic AMP and ocadaic acid, a protein phosphatase inhibitor, suppressed autophagic segregation. Interestingly, the activity of some rab proteins may also be inhibited by phosphorylation (Bailly et al. 1991, Pryer et al. 1992).

2.4.7 Experimental inhibition of autophagy

Autophagy can be inhibited by various drugs, and these have been widely applied in studies on the mechanisms and regulation of autophagy. Inhibitors exist for the sequestration, enzyme delivery and degradation stages. Gordon and Seglen (1982) have developed a biochemical method to measure the different stages of the autophagic pathway. A radioactively labelled saccharide, sucrose or raffinose, is first introduced into the cytoplasm of electropermeabilized isolated hepatocytes. After resealing, the accumulation of the non-hydrolyzable sugar in sedimentable vesicles (autophagic vacuoles) can then be measured from homogenates or electrodisrupted cells. Delivery of lysosomal hydrolases to the sugar-containing vesicles can be measured using lactose which is degraded in lysosomes (Høyvik et al. 1986).

Seglen and Gordon (1982, 1984) found a specific inhibitor of autophagic sequestration, 3-methyladenine. This drug as good as completely prevents the formation of new autophagosomes in isolated hepatocytes (Seglen et al. 1986). In fibroblasts, 3-methyladenine inhibits both autophagic and endocytic protein degradation (Hendil et al. 1990). Phalloidin, a stabilizer of F-actin filaments, has also been shown to hinder autophagosome formation (Ueno et al. 1990). Cycloheximide (Kovács et al. 1975, Kovács 1983) and amino acids (Seglen & Gordon 1984) have also been used to inhibit autophagic segregation.

A reduction in temperature blocks the formation of new autophagosomes. No significant sequestration occurs below +20°C (Kovács & Kovács 1980, Gordon et al. 1987). Several transport blocks also exist on the exocytic and endocytic pathways (Kuismanen & Saraste 1989). Newly-synthesized proteins are not transported from the TGN to the cell surface below +20°C (Saraste & Kuismanen 1984). At +15°C, the proteins accumulate in a compartment between the ER and Golgi apparatus (Kuismanen & Saraste 1989). On the endocytic pathway, the transport of endocytic markers from early endosomes to the PLC is blocked at +20°C (Dunn et al. 1980, Marsh et al. 1986).

In 1974, the microtubule inhibitor vinblastine was found to induce an accumulation of autophagic vacuoles both in vivo and in vitro (Arstila et al. 1974, Hirsimäki et al. 1975, Kovács et al. 1975). The accumulation was generally thought to be due to an increase in the formation of new autophagosomes. Five years later, Marzella and Glaumann (1980b) and Kovács et al. (1982) found that vinblastine inhibited protein degradation. Hence, Kovács et al. (1982) proposed that vinblastine acts by inhibiting the fusion between autophagosomes and lysosomes, which leads to the accumulation of the former. Vinblastine was indeed found to increase the half-life of autophagic vacuoles from 6 to 27 min in several tissues (Kovács et al. 1988, Kovács & Réz 1989). Vinblastine has also been found to prevent the degradation of autophaged lactose microinjected into isolated hepatocytes (Høyvik et al. 1986). In hepatocytes, vinblastine accumulates mainly double-membrane limited autophagic vacuoles (Hirsimäki & Pilström 1982). In contrast, in Ehrlich ascites cells and 3T3 fibroblasts vinblastine causes an accumulation mainly of older, single-membrane-limited autophagic vacuoles (Reunanen & Nykänen 1988, Miettinen & Reunanen 1991). Furthermore, two other microtubule inhibitors, nocodazole and griseofulvin, cause no accumulation of autophagic vacuoles or lysosomes in Ehrlich ascites cells (Reunanen et al. 1988b). These results suggest that microtubule depolymerization does not prevent lysosomal enzyme delivery in all cell types.

The degradation of autophagically segregated material can be retarded by using enzyme inhibitors or drugs which affect the lysosomal pH. Leupeptin, an inhibitor of lysosomal cysteine proteinases, inhibits protein degradation and induces accumulation of autophagic vacuoles (Furuno et al. 1982, Henell & Glaumann 1984). Chloroquine is a lysosomotropic agent; it accumulates in lysosomes and raises their pH. The carboxylic ionophore monensin also raises the lysosomal pH (Seglen 1983). These drugs also accumulate autophagic vacuoles in cells (Wisner-Gebhart et al. 1980, Grinde 1983). Bafilomycin A_1, a specific inhibitor of vacuolar-type H^+-ATPase, has also been shown to inhibit lysosomal degradation (Yoshimori et al. 1991).

2.5 Non-lysosomal degradation

2.5.1 Degradation in the ER

Newly-synthesized proteins fold and assemble in the RER lumen. Folding is assisted by the ER luminal proteins, protein disulfide isomerase (PDI) and immunoglobulin binding protein (BiP). However, improperly folded and incompletely assemled proteins are degraded before they reach the Golgi apparatus (reviewed by Klausner & Sitia 1990, Sitia & Meldolesi 1992, Hauri & Schweizer 1992). Incompletely assembled T-cell receptor subunits were degraded in transfected fibroblasts in a pre-Golgi compartment by a pathway which was insensitive to drugs inhibiting lysosomal degradation (Lippincott-Schwartz et al. 1988). In permeabilized cells, ER degradation occurred in the absence of cytosol and ATP, both prerequisites for vesicle-mediated transport, which suggests that the degradation occurs in the ER itself (Stafford & Bonifacino 1991). The newly-discovered compartment between the ER and Golgi, intermediate or salvage compartment (Schweizer et al. 1990, Saraste & Svensson 1991), may be the site of final pre-Golgi degradation (Wikström & Lodish 1991). ER degradation also regulates cholesterol biosynthesis. The rate-limiting enzyme of cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A reductase, is rapidly degraded when the cholesterol level is high. As the cholesterol level falls, the half-life of the enzyme increases (Gil et al. 1985, Klausner & Sitia 1990).

Proteins may also be sent from the ER directly for lysosomal degradation. Insoluble protein aggregates accumulating in the RER lumen in pancreatic acinar cells after $CoCl_2$ treatment were sequestrated, surrounded by the RER membrane, in autophagosomes and degraded lysosomally (Tooze et al. 1990). However, similar protein aggregates induced in thyroid hormone -secreting cells were degraded by a hitherto unknown process. The RER cistern containing aggregates first shed its ribosomes, then acquired lysosomal membrane proteins, and finally lysosomal enzymes (Noda & Farquhar 1992).

2.5.2 Ubiquitin-mediated degradation

Ubiquitin is a 76 -amino acid protein found in all eucaryotic cells. The bulk of abnormal and short-lived regulatory proteins are degraded in the cytoplasm by a ubiquitin-mediated route (reviewed by Finley & Varshavsky 1985, Jentsch et al. 1990, Hershko & Ciechanover 1992). Proteins are first linked to one or more ubiquitin molecules by three ATP -

dependent cytoplasmic enzymes. The selectivity of ubiquitination is based on the amino-terminal residue of the proteins (Bachmair et al. 1986). The conjugated proteins are then degraded by a specific ATP-dependent protease complex (Hershko & Ciechanover 1992).

Recent studies have revealed that ubiquitin-protein conjugates are enriched in the lysosomal vesicles of fibroblasts (Doherty et al. 1989, László et al. 1990, Lenk et al. 1992). The morphological results suggest that the conjugates may be taken into the lysosomes by microautophagy (László et al. 1990). However, it is not clear whether the enrichment is due to selective uptake or retarded degradation (Ueno & Kominami 1991). It was only recently that cells containing a thermolabile mutant of the ubiquitin -activating enzyme, E1, were found to be defective in stress-induced degradation of intracellular proteins at a restrictive temperature (Gropper et al. 1991). In ultrastructural studies, these cells were observed to posses large numbers of acidic autophagic vacuoles which contained lysosomal enzymes but were presumably unable to mature into residual bodies (Lenk et al. 1992). The result thus suggests that E1 or protein ubiquitination may be associated with autophagic degradation. These results indicate a possible connection between the lysosomal and ubiquitin-mediated degradation pathways.

3 AIM OF THE STUDY

The aim of the present study was to investigate, using electron microscopical methods, the maturation of autophagosomes into autolysosomes, in particular to elucidate the structure of the limiting membranes and the route of lysosomal enzyme delivery. The specific topics were as follows:

1. Membrane protein and cholesterol content of the membranes of autophagosomes, autolysosomes, residual bodies and lysosomes in Ehrlich ascites cells (I) and mouse hepatocytes (II).

2. Effect of the microtubule inhibitor vinblastine on autophagy in Ehrlich ascites cells (III).

3. Roles of the cation-independent mannose 6-phosphate receptor and acidification in the delivery of lysosomal enzymes to autophagosomes in cultured rat fibroblasts (IV, V).

4. Roles of the the prelysosomal compartment and trans-Golgi network in the delivery of lysosomal enzymes to autophagosomes in cultured rat fibroblasts (V).

4 SUMMARY OF MATERIALS AND METHODS

4.1 Experimental animals

Male (II) or female (I, III; culturing of Ehrlich ascites cells) NMRI mice, 2-5 months old and weighing 22-40 g, were used in the experiments. The animals were starved for 17 h before the cell fractionation experiments (II). Vinblastine was used to accumulate autophagic vacuoles in hepatocytes (II), and was administered to mice intraperitoneally (50 mg/kg) 2 or 3 h before decapitation.

4.2 Cell lines

Ehrlich ascites cells were grown in the peritoneal cavities of NMRI mice for 6 or 7 days (I, III). Fibroblasts isolated from rat embryos were used for the experiments between cell generations 5 and 10 (IV, V). The cells were cultured (+37°C, 10% CO_2) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Subconfluent and growing cultures were used for the electron microscopy and cell fractionation, and immunofluorescence experiments, respectively.

4.3 Endocytic markers

The markers were diluted in serum-free DMEM containing 25 mM Hepes buffer, pH 7.4 (IV, V). Rat fibroblasts were incubated with cationized ferritin (CF) (100-250 μg/ml) at +4°C for 10 min to allow attachment to the plasma membrane. The cells were then rinsed with phosphate-buffered saline (PBS) and chased for 5 min - 6 h at +37°C to allow internalization of CF. BSA-coated gold particles (14 nm) were prepared according to Slot and Geuze (1985). Gold particles (OD_{520} was 0.5) were added to the cells for 2 h. The cells were then rinsed with PBS and either fixed immediately or chased for 2 or 24 h at +37°C. For the electron microscopy, the cells were incubated with HRP (10 mg/ml) at +37°C for 15-120 min. The cells were then rinsed with PBS and either fixed immediately or chased for 15 min - 4 h. For the cell fractionation experiments, the cells were incubated with HRP (2-4 mg/ml) at +37°C for 5 min, washed on ice with ice-cold PBS containing BSA and finally with PBS, and chased in serum-free DMEM at +37°C for 0-60 min.

4.4 Microscopical methods

4.4.1 Conventional electron microscopy and lipid cytochemistry

The samples were fixed in 1-2.5% glutaraldehyde in phosphate or cacodylate buffer, pH 7.4 or 7.5, and post-fixed in 1% OsO_4 (I, II, IV, V). In III, Ehrlich ascites cells were fixed in a mixture of 1% OsO_4 and 4% glutaraldehyde (1+1). The samples were then dehydrated in a series of ethanol and embedded in Epon (LX-112). Thin sections were cut, mounted on copper grids and stained with uranyl acetate and lead citrate.

Membrane cholesterol was detected using filipin (100-300 μg/ml) which was added to the fixation medium (I, II) (Elias et al. 1979). Unsaturated fatty acids were detected with imidazole-buffered 2% OsO_4 (I) (Angermüller & Fahimi 1982).

4.4.2 Freeze fracture

For freeze-fracture, Ehrlich ascites cells (I) and mouse liver samples (II) were fixed as described above, infiltrated with glycerol, frozen in liquid Freon 22 cooled with liquid nitrogen, and freeze-fractured at -120°C. The platinum shadowing was performed at an angle of 40° or 45°.

For the determination of intramembrane particle density, the surface area of each membrane fracture face was determined by point counting from printed photomicrographs (final magnification was X127,750 in I and X80,000 in II). Particle diameters were measured from the micrographs using a measuring magnifier. The magnification was calibrated using a calibration grid (Balzers, crossed lines grating, 54,000 lines per inch).

4.4.3 Acid-phosphatase and HRP cytochemistry

Acid phosphatase was demonstrated (V) using the modified lead-based method of Barka and Anderson (1962) with sodium-β-glycerophosphate as substrate (Miettinen & Reunanen 1991).

Endocytosed HRP was visualized as described in Griffiths et al. (1989) using 0.01% hydrogen peroxide and 0.1% diaminobenzidine in 0.05 M Tris-HCl buffer, pH 7.4, at room temperature for 4-8 min.

4.4.4 Morphometric methods

Morphometric analysis of subcellular fractions (II) and Ehrlich ascites cells (III) was performed by point counting (Weibel 1969). In II, thin sections were cut from the organelle fractions. The volume fractions of different organelles were determined from printed photomicrographs (final magnification was X26,000). In III, diapositives were prepared from the micrographs (primary magnification was X5,000). Calculations were performed by projecting the diapositives onto a double-square test lattice (Weibel & Bolender 1973). Stereological estimates were expressed as volume densities and related to the cytoplasmic volume. The number of profiles per unit area was determined by counting the vacuoles occurring on the tested area. Mean cell diameter was measured from light-microscopic sections cut from the LX-112 blocks. This was used to estimate mean cell volume.

4.4.5 Cryosectioning and immunoelectron microscopy

The monolayers of rat fibroblasts (IV, V) were fixed in 4% paraformaldehyde, 0.1% glutaraldehyde, 0.01% azide, and 7.5% sucrose in PBS at room temperature for 60 min. The cells were then infiltrated with 2.1 M sucrose, and frozen in liquid nitrogen. Ultrathin frozen sections were cut

at -90°C as described in Griffiths et al. (1984). Double immunolabelling of the sections was carried out according to Geuze et al. (1981) and Tokuyasu (1986) using protein A-coated gold particles of two sizes (5 or 6 and 10 nm in diameter) prepared according to Slot and Geuze (1985). The labelled sections were contrasted and embedded on ice in 0.3% uranyl acetate in 1.5% methylcellulose.

For quantitation of labelling, early autophagic vacuoles were micrographed from the best sections of each sample. The number of gold particles per vacuole (IV) or square micro metre (V) was calculated from micrographs printed at a final magnification of X65,000. The size of the autophagic vacuole profiles and the intensity of the background labelling over the nucleus were determined by point counting.

4.4.6 Immunofluorescence

Fibroblasts were fixed (IV, V) in 4% paraformaldehyde in PBS, at room temperature for 45 min. The cells were permeabilized with Triton X-100 and incubated in 5% fetal calf serum in PBS for 15 min and then with the primary antibodies in the same solution for 60 min. After rinsing, the cells were incubated with a fluorescently labelled secondary antibody for 30 min. The monolayers were mounted in glycerol containing para-phenylenediamine.

4.4.7 Antibodies

The following antibodies were used (IV, V): gammaglobulin fraction of a rabbit antiserum against bovine cation-independent mannose 6-phosphate receptor (Marjomäki et al. 1990); rabbit antiserum against dinitrophenol (ICN ImmunoBiologicals); and rabbit antiserum against mouse cathepsin L (the generous gift of Dr. Michael Gottesman, National Cancer Institute, Bethesda, MD, USA; Gottesman & Gabral 1981). The specificities of the antibodies against the 215 kDa MPR and cathepsin L were verified with immunoblotting (Marjomäki et al. 1990, and unpublished results from the present study). Normal rabbit serum, normal rabbit IgG (Dakopatts), or rabbit antiserum against bovine serum albumin (Bio Yeda) were used as control antibodies. Anti-rabbit IgG-FITC was used as a secondary antibody in immunofluorescence stainings.

4.5 Biochemical methods

4.5.1 Isolation of autophagic vacuoles and lysosomes

Autophagic vacuoles were isolated from the livers of vinblastine-injected mice, and lysosomes from the livers of untreated mice (II). The fractionations were made from the mitochondrial-lysosomal and light mitochondrial fractions, respectively, using discontinuous metrizamide gradients (Wattiaux et al. 1978, Wattiaux & Wattiaux-de Coninck 1983, Marzella et al. 1982). The fractions were characterized biochemically and morphometrically. Marker enzyme activities for lysosomes (acid phosphatase), ER (glucose 6-phosphatase), plasma membrane (5'nucleotidase), and mitochondria (succinate dehydrogenase) were assayed.

4.5.2 Subcellular fractionation

Post nuclear supernatants of fibroblasts were prepared (V) and mixed with 27% Percoll as described in Griffiths et al. (1990). The gradients were centrifuged at +4°C for 120 min at 30,000 g. Fractions of 0.5 ml were then collected beginning from the bottom of the tubes. Marker enzymes for lysosomes and the PLC (β-hexosaminidase and tartrate-inhibitable acid phosphatase) and HRP (endosytic marker) were assayed from the fractions. MPR (marker of the PLC) was detected by immunoblotting.

4.5.3 Protein degradation

Proteins of Ehrlich ascites cells (III) were labelled by injecting [^{14}C]valine i.p. into the mice (2 μCi/10 g) 24 h before the experiments. Unlabelled valine was included in the incubation medium to prevent reincorporation of [^{14}C]valine. Protein degradation was expressed as the net release of acid-soluble radioactivity during the incubation period (Seglen et al. 1979). It was calculated as a percentage of total protein-incorporated radioactivity (Seglen 1978) at the beginning of incubation.

4.5.4 Immunoblotting

Fibroblasts (V) were lysed and extracted in a buffer containing 0.05 M Tris-HCl, pH 7.4, 0.5% Triton X-100, 0.05% SDS. The samples were separated in polyacrylamide gel electrophoresis, and proteins were transferred electrophoretically to nitrocellulose. The blots were immunolabelled for cathepsin L or MPR, using biotin-conjugated anti-rabbit IgG and either streptavidin or Extravidin conjugated alkaline phosphatase. Alkaline phosphatase was visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate.

4.5.5 Metabolic labelling and immunoprecipitation

Fibroblasts were treated with methionine-free minimum essential medium containing 10% FCS dialyzed against PBS, and 10 mM Hepes, pH 7.4, for 15 min, and then metabolically labelled with [^{35}S] methionine in the same medium for 30 min. The cells were then chased in serum-free minimum essential medium containing 1.5 mg/ml nonradioactive methionine for 1 or 2 h. After chasing, the dishes were placed on ice, and the cells were lysed and extracted in a buffer containing 0.05 M Tris-HCl, pH 7.4, 0.150 M NaCl, 1% Triton X-100, 0.05% SDS, 1 mM EDTA. Cathepsin L was immunoprecipitated from the cell extracts and chase media, using cathepsin L antiserum and protein A-Sepharose. The latter was washed in a buffer containing 0.05 mM Tris-HCl, pH 7.4, 0.150 mM NaCl, 0.5% Triton X-100, 0.1% SDS, 1 mg/ml BSA, and finally in the same buffer without BSA. The samples were then mixed with gel sample buffer, reduced, and separated in polyacrylamide gels. The gels were dried under a vacuum and exposed to X-ray film.

4.6 Statistical methods

Student's t-test and analysis of variance were used for testing significances between the means in the morphometric analysis (III). G-test of independence, chi-square test of homogeneity, Kruskal-Wallis one way analysis of variance and Mann-Whitney U-test were used in the quantitation of immunolabelling (IV, V).

5 REVIEW OF THE RESULTS

5.1 Structure of the autophagic-vacuole membrane in Ehrlich ascites cells and hepatocytes

Ehrlich ascites cells and mouse hepatocytes are both well characterized models for studies on autophagy (Hirsimäki et al. 1975, Hirsimäki & Pilström 1982). The structure of the membranes of early and later autophagic vacuoles and lysosomes was compared in the present study (I, II). Freeze fracture technique was used to visualize membrane surfaces. Autophagic vacuoles were accumulated using vinblastine in Ehrlich ascites cells (0.1 mM, 30 min; I) and mouse hepatocytes (50 mg/kg, 2 or 3 h; II). Early autophagic vacuoles were accumulated in Ehrlich ascites cells with iodoacetate (2.5 mM), an inhibitor of glycolysis (I) (Hirsimäki et al. 1984, Reunanen & Nykänen 1988). Lysosome-rich subcellular fractions of liver were used to study lysosomal membranes.

Vinblastine caused an accumulation of autophagic vacuoles in Ehrlich ascites cells (I) and mouse hepatocytes (II). In Ehrlich ascites cells, the vacuoles consisted mainly of older, single-membrane limited forms, whereas in mouse hepatocytes, both double- and single-membrane limited forms were found. Iodoacetate raised the number of double-membrane limited vacuoles in Ehrlich ascites cells. The membranes of these vacuoles sometimes showed attached ribosomes (I, Fig. 2a). In freeze-fracture replicas, early autophagic vacuoles were defined as round vacuoles in which an inner membrane parallel with the outer one was clearly visible (I, Figs 3a, 5a; II, Fig. 1a-d). Later vacuoles were more irregular-shaped and possessed only some remnants of the inner membranes (I, Figs 3b-e, 4c, 5c; II, Fig. 2a-c). In replicas of Ehrlich ascites cells, small round vesicles were also observed (I, Figs 3d, e, ad). Their size and shape

suggested that they correspond to electron-dense residual body -type vacuoles (I, Fig. 2d). Lysosomes were difficult to identify in replicas made of intact liver tissue. Therefore, replicas of isolated lysosomes were used.

5.1.1 Unsaturated fatty acids and cholesterol

Imidazole-buffered OsO_4 was used to detect unsaturated fatty acids (Angermüller & Fahimi 1982) in Ehrlich ascites cells (I). Positive staining is observed in thin sections as increased electron density. Membrane cholesterol was specifically labelled using filipin (I, II), which induces characterictic membrane deformations by forming complexes with unesterified cholesterol (Elias et al. 1979). Positive labelling is observed in thin sections and freeze-fracture replicas as 20-30 nm pits and protrusions. The molar ratio of cholesterol to phospholipids must be at least 1:20 to produce visible lesions (Elias et al. 1979). Because filipin penetrated the tissue blocks poorly, subcellular fractions were used for the filipin labelling of liver organelles (II).

Imidazole-buffered OsO_4 stained the membranes of early, double-membrane limited autophagic vacuoles in Ehrlich ascites cells. Curved cisternae of ER (possibly in the process of sequestrating cytoplasm) were also stained. The membranes of older, single-membrane limited vacuoles were not stained (I, Fig. 1a-c).

Filipin labelling was studied in thin sections and freeze-fracture replicas (I, II). The membranes of some double-membrane limited autophagic vacuoles did not show any filipin labelling. Since in thin sections the contents of these vacuoles appeared unaltered, the vacuoles were judged to be nascent (I, Figs 2a, b, 4a; II, Fig. 5a). However, the membranes of most, both double- and single-membrane limited, vacuoles were labelled by filipin. The membranes visible inside the vacuoles were also often labelled (I, Figs 2c, 3a-e, 4b, c; II, Figs 5b, 6a, b). In Ehrlich ascites cells, the membranes of the putative residual bodies were labelled weakly or not at all (I, Figs 2d, 3e, 4d), whereas the membranes of isolated mouse-liver lysosomes were labelled heavily (II, Fig. 7a, b). Thus, the content of unsaturated fatty acids appeared to decrease from early to later autophagic vacuoles, whereas the cholesterol content increased.

5.1.2 Membrane proteins: intramembrane particles in freeze-fracture replicas

The occurrence of membrane proteins was studied by counting the density of intramembrane particles in freeze-fracture replicas. These corre-

spond to membrane proteins (Rash et al. 1981, Borovyagin et al. 1985). The approximate protein content in different membranes can thus be compared. The freeze-fracture nomenclature was established by Branton et al. in 1975. The fracture face which remains attached to the cytoplasm is called the protoplasmic or P face, and the other fracture face remaining attached to the organelle lumen is the exoplasmic or E face. The density of intramembrane particles was counted on the membranes of iodoacetate-induced double-membrane limited, and single-membrane limited, vacuoles as well as on the ER in Ehrlich ascites cells (I, Table 1), and on the membranes of double-membrane limited vacuoles and isolated lysosomes in mouse liver (II, Table 3). Table 1 presents a summary of the membrane particle densities in autophagic vacuoles and lysosomes.

TABLE 1. Intramembrane particle densities (no/μm^2±SE) on the membranes of autophagic vacuoles and lysosomes.

	Early AVs	Later AVs	Lysosomes
Ehrlich ascites cells			
PF	2526±237	2783±160	
EF	2502±200		
Hepatocytes			
PF	328±53		1440±42
EF	310±41		688±31

AV, autophagic vacuole; PF, P face; EF, E face. In Ehrlich ascites cells, the P-face density of early AVs and E-face density are from iodoacetate-treated cells. The E - face density was not calculated separately for early and later AVs.

In Ehrlich ascites cells, the membranes of double-membrane limited autophagic vacuoles showed uniformly distributed intramembrane particles on their P fracture face (I, Fig. 5b). The membranes of these vacuoles resembled those of the ER (I, Fig. 5a, b). Older, apparently single-membrane limited vacuoles contained more particles on their P fracture face (I, Fig. 5c). Moreover, the P-face particles were larger in the older vacuoles than in the early ones. The percentage of particles over 7 nm in diameter was 27 and 43- 49% in the early and later vacuoles, respectively (I, Table 1). The limiting membranes also showed intramembrane parti-

cles on their E fracture faces (I, Fig. 5d, e). However, the membranes of the putative residual body -type vacuoles were usually particle-free (I, Figs 4d, 5f).

In mouse hepatocytes and autophagic-vacuole fractions, the membranes of all autophagic vacuoles contained only few or no intramembrane particles. The membranes of double-membrane limited vacuoles were usually completely particle-free (II, Fig. 1a, b, d). Sometimes, fusion profiles of particle-rich vesicles and smooth autophagic vacuoles were observed (II, Fig. 1c). The membranes of apparently single-membrane limited, irregular-shaped vacuoles usually showed some particles (II, Fig. 2a-c). The membranes of ER contained more particles than the autophagic vacuoles (II, Fig. 3). Many uniformly distributed particles were also visible on the membranes of isolated lysosomes (II, Fig. 4a-d). Thus, the density of intramembrane particles appears to increase as autophagic vacuoles mature into degradative lysosomal vacuoles. The size of the particles also increases simultaneously.

5.2 Microtubules and autophagy in Ehrlich ascites cells

The aim was to study the role of microtubules in the delivery of lysosomal enzymes to autophagic vacuoles. Microtubules were disrupted with vinblastine. Electron-microscopic morphometry was used to study whether vinblastine accumulated autophagosomes or autolysosomes in Ehrlich ascites cells, and whether leucine and histidine (the amino acids which inhibit sequestration, Seglen & Gordon 1984), as well as 3-methyladenine (a specific inhibitor of segregation, Seglen et al. 1986), were able to prevent the accumulation (III).

The cells were divided into six groups, and a preincubation of 30 min was performed before vinblastine addition (0.1 mM):

1) No additions;
2) Preincubation without additions, incubation with vinblastine;
3) Both incubations with leucine and histidine (10 mM each);
4) Both incubations with 3-methyladenine (20 mM);
5) As group 3, but incubation including vinblastine; and
6) As group 4, but incubation including vinblastine.

Aliquots of 1-3 ml were taken from the cell suspensions after 2, 30, 60, and 120-min incubations for the electron microscopy, and after 0 and 120-min incubations for the measurement of protein degradation.

The criteria used to classify the autophagic-lysosomal vacuoles in the morphometric analysis are described in III under Results and in Fig. 1. Double-membrane limited autophagosomes were surrounded by two membranes and contained morphologically intact cytoplasm (III, Fig. 1A); electron-lucent vacuoles (later autophagic vacuoles) were surround-

ed by a single membrane and contained partially degraded cytoplasmic material (III, Fig. 1B-D); and dense vacuoles (residual body -type vacuoles) were limited by a single membrane and contained electron-dense material (III, Fig. 1D, E).

Vinblastine significantly increased the cytoplasmic volume density and number of electron-lucent vacuoles in 30 and 60 min, respectively. The number of autophagosomes remained at the control level. After a 120-min incubation, the volume density and number of dense vacuoles were both significantly smaller in the vinblastine-treated cells as compared with the control cells. Leucine and histidine significantly retarded but did not prevent the vinblastine-induced accumulation of electron-lucent vacuoles. However, 3-methyladenine prevented the accumulation almost completely (III, Tables I, II). The viability (nigrosin staining) and volume of the cells did not change during the 120-min incubation (III, Table III).

Protein degradation was measured to detect the degradation of cytoplasmic material in autophagic vacuoles. Degradation was measured as a release of radioactive valine from prelabelled long-lived endogenous proteins. The results of the measurements are presented in III, Table IV. Vinblastine slightly inhibited the rate of protein degradation but the change was statistically insignificant. Thus, although vinblastine accumulated later autophagic vacuoles, the rate of protein degradation did not increase. This suggests that the accumulation was caused by retarded degradation in the later autophagic vacuoles.

5.3 MPR, acidification, and cathepsin L delivery to autophagic vacuoles in fibroblasts

The aim was to study the route of enzyme delivery to autophagosomes. Cryoimmuno elecron microscopy was used to detect MPR which recycles between the PLC and TGN in the multiple delivery cycles of lysosomal enzymes. In fibroblasts, over 90% of MPR is located in the PLC in steady state (Griffiths et al. 1988, 1990). Cathepsin L, a lysosomal proteinase, was used as a marker of the PLC and mature lysosomes. Acidic compartments were labelled using an acidotropic amine, 3-(2,4-dinitroanilino)-3'-amino-N-methyl-dipropylamine (DAMP), (100 μM, 30 min) which can be detected immunocytochemically with anti-dinitrophenol (Anderson et al. 1984). Cultured fibroblasts were used because the role of MPR in enzyme delivery in these cells is well documented (von Figura & Hasilik 1986, Griffiths et al. 1988). Cationized ferritin (CF) was used as a marker of adsorptive endocytosis.

The experiments (IV) were performed to answer three questions: firstly, whether the enzymes are delivered to early autophagic vacuoles

by MPR-positive or negative vesicles; secondly, whether enzyme delivery occurs simultaneously with acidification; and thirdly, whether acidification is obligatory for enzyme delivery. To induce autophagy, the cells were incubated in a serum-free medium for 30 min (Mitchener et al. 1976).

5.3.1 Distribution of MPR, cathepsin L and DAMP in fibroblasts

The most intense labelling for MPR, cathepsin L and DAMP was found in vesicles which contained tightly-packed lamellar or concentric membrane figures or tubular-vesicular membrane material (IV, Fig. 1a, b). The vesicles were also often positive for endocytosed CF (IV, Fig. 1b), and thus correspond to the PLC. The Golgi stacks were usually not labelled, whereas tubules and vesicles associated with the stacks occasionally contained MPR, cathepsin L (IV, Fig. 1c) and DAMP (not shown). Cathepsin L, DAMP and occasional MPR labelling were also found in more electron-dense vesicles (IV, Fig. 1a, d) which contained large amounts of the endocytic marker (IV, Fig. 1d). These structures correspond to mature lysosomes.

5.3.2 MPR, cathepsin L and DAMP labelling in autophagic vacuoles

Early autophagic vacuoles were frequently observed in fibroblasts incubated in a serum-free medium for 30 min (IV, Fig. 2a-c), but only rarely in those incubated with 10% serum. In cryosections, early autophagic vacuoles were identified as round, oval or irregular-shaped vesicles containing material closely resembling the surrounding cytoplasm (IV, Figs 3, 4a-f). The limiting membranes of the vacuoles were poorly visible or difficult to distinguish from the membrane structures in the surrounding cytoplasm. Usually, an electron-translucent layer was visible between the limiting membrane and contents (IV, Figs 3, 4a-f). Thus, since the double limiting membrane is poorly recognizable and ribosomes are not visible at all in cryosections, the only available marker for the identification of autophagic vacuoles was, as stated above, 'material resembling the cytoplasm' inside the vacuoles.

Immunolabelling for MPR, cathepsin L and DAMP was observed in the early autophagic vacuoles (IV, Table 1; Figs 3, 4a-d). About one-fifth (21–24%) of the vacuoles showed moderate to weak labelling for both MPR and cathepsin L, DAMP and cathepsin L, or DAMP and MPR. The rest of the early vacuoles showed weak labelling for one only of the antibodies used in the double labelling experiments, or were unlabelled.

In cryosections, endocytosed ferritin (10-min binding and 3-h uptake) was not observed in the early autophagic vacuoles. The results suggest that at least part of cathepsin L in autophagic vacuoles originates from the MPR-positive compartments, i.e. the PLC or TGN.

5.3.3 Effect of monensin on the labelling of early autophagic vacuoles

To study the role of acidification in the delivery of cathepsin L to autophagic vacuoles, monensin (10 μM) was used to raise the pH of acidic organelles (Seglen, 1983). Immunofluorescence staining showed that DAMP did not accumulate in fibroblasts when monensin was present in the medium (IV, Fig. 5b). However, the presence of monensin during the induction of autophagy did not change the labelling of early autophagic vacuoles for MPR and cathepsin L (IV, Table 1; Fig. 4e). Monensin treatment raised the proportion of early autophagic vacuoles labelled for cathepsin L but not for DAMP. However, monensin treatment did not change the number of particles detecting MPR or cathepsin L in autophagic vacuoles (IV, Table 2; Fig. 4f). Thus, acidification is not obligatory for cathepsin L delivery.

5.4 Endosomes and enzyme delivery to autophagic vacuoles in fibroblasts

The role of the PLC in enzyme delivery was studied using endocytic markers (V). The cells were labelled with a marker and chased for varying time periods. Autophagy was induced by incubation in serum-free DMEM for 1 or 2 h before fixation (before, during and/or after the endocytosis). Leupeptin (250 μM), an inhibitor of cysteine proteinases, was added to the serum-free DMEM for some of the culture dishes. This facilitated the morphological identification of autophagic vacuoles.

Three different endocytic markers were used: CF as a marker of non-selective adsorptive endocytosis; colloidal gold particles coated with bovine serum albumin (BSA-gold) as a particulate marker of fluid-phase endocytosis; and horseradish peroxidase (HRP) as a soluble marker of fluid-phase endocytosis. CF and BSA-gold are readily visible in thin sections, whereas HRP was visualized by a cytochemical staining for peroxidase activity. The labelling protocols necessary to chase the markers into autophagic vacuoles were first established using Epon-embedded specimens. The subcellular locations of the markers with these protocols were then determined by cryoimmunoelectron microscopy (CF and BSA-gold) or cell fractionation (HRP).

5.4.1 Cationized ferritin

Autophagic vacuoles did not contain CF when the cells were chased for 5-15 min after the 10-min labelling at +4°C, and only occasionally after a 30-45 min chase. CF was frequently found in autophagic vacuoles after a chase of 1-6 h (V, Fig. 1A-D). The subcellular localization of CF was studied after chases of 1, 2 and 3 h. Cryosections were prepared and double-labelled for MPR and cathepsin L. After internalization for 1 h, CF was found in the PLC (MPR-enriched, cathepsin L -positive structure) as well as in peripheral vesicles lacking MPR and cathepsin L (V, Fig. 2A) which probably correspond to early endosomes. Most lysosomes (cathepsin L -positive and MPR-negative vesicles) did not contain CF (V, Fig. 2B). With longer internalization periods, more CF accumulated in lysosomes (V, Fig. 2C). After a 3-h internalization, CF was present in many PLC profiles as well as in lysosomes (IV, Fig. 1 b, d).

5.4.2 BSA -coated gold particles

Gold particles were detected in a few autophagic vacuoles after a 2-h uptake at +37°C (V, Fig. 3A), and more frequently after a 2-h uptake followed by a 2 or 24-h chase (V, Fig. 3B, C). Acid phosphatase, a marker of the TGN, PLC and lysosomes, was demostrated cytochemically. In all uptake-chase experiments, BSA-gold was detected in both acid phosphatase -positive and negative vesicles, and both were observed in fusion profiles with autophagic vacuoles (V, Fig. 3A-C). Earlier studies have shown that protein-coated gold particles are transported to the PLC in 2 h in fibroblasts (Griffiths et al. 1988). In cryosections labelled for MPR and cathepsin L, BSA-gold was found in both the PLC and lysosomes after both 2 and 24-h chase periods.

In some experiments, the PLC and lysosomes were first labelled with BSA-gold (2-h uptake and 24-h chase), and early endosomes were then labelled with CF (10-min binding and 15-min chase). In these conditions, the two markers were never observed inside the same vesicles. BSA-gold was detected in autophagic vacuoles whereas CF was not.

5.4.3 Horseradish peroxidase

HRP activity was not detected in autophagic vacuoles after a 15-min internalization (V, Fig. 4A) but was found in small tubules and vesicles located in the periphery of the cells. After 30-min uptake with or without a

15-min chase, HRP was found in a few autophagic vacuoles (V, Fig. 4B, C). After internalization for 30-120 min and a 1-h chase, HRP was frequently detected in autophagic vacuoles (V, Fig. 4D). With this labelling protocol, the number of HRP-positive autophagic vacuoles increased with longer internalization periods (when HRP filled an increasing proportion of the endosomal/lysosomal compartments). HRP-positive vesicles were frequently detected in contact with early autophagic vacuoles containing morphologically unaltered cytoplasm (V, Fig. 4E, F). However, the reaction product masked the contents of most later autophagic vacuoles and made them difficult to identify (V, Fig. 4B).

The transport of HRP from endosomes to lysosomes was followed using cell fractionation of the post-nuclear supernatants on Percoll gradients (V, Fig. 5). In fibroblasts, since the bulk of MPR is located in the PLC (Griffiths et al. 1988), the location of MPR in the gradient indicates the PLC. MPR (215 kDa) was detected by immunoblotting from the pooled fractions (V, Fig. 5B). β-Hexosaminidase, which is located in lysosomes and to a lesser extent in the PLC, and acid phosphatase, which is located in the TGN, PLC and lysosomes, were also measured (V, Fig. 5A). The results showed that HRP reached the PLC in 30 min, at the same time as it was first detected in autophagic vacuoles. After 30-120 min of internalization, followed by a 1-h chase, when many autophagic vacuoles were HRP-positive, HRP would localize to both the PLC and lysosomes (V, Fig. 5C-E).

5.5 TGN and cathepsin L delivery to autophagic vacuoles in fibroblasts

To study whether MPRs transport newly-synthesized cathepsin L directly from the TGN to autophagosomes (V), two inhibitors of MPR-mediated transport, tunicamycin (2 μg/ml) and chloroquine (50 μM), were used. Tunicamycin inhibits the glycosylation of lysosomal enzymes, thus preventing synthesis of the mannose 6-phosphate recognition marker (Imort et al. 1983). Chloroquine prevents the dissociation of enzymes from the receptors in the PLC, presumably by raising the pH in acidic organelles (Seglen 1983, Kornfeld & Mellman 1989). Occupied receptors accumulate in large vacuolar endosomes which can be detected by immunofluorescence staining (Brown et al. 1984). Both drugs prevent MPR-mediated targeting of lysosomal enzymes from the TGN to the lysosomal system: tunicamycin by preventing the binding of enzymes to MPRs, and chloroquine by causing a deficiency of free receptors. In both cases, the enzymes are secreted via the constitutive secretory pathway as unprocessed precursors.

The effect of tunicamycin on the glycosylation of cathepsin L was studied by immunoblotting and metabolic labelling followed by immunoprecipitation (V, Fig. 6). Immunoblotting of cathepsin L from the control cells revealed the three forms of the enzyme: the 39-kDa precursor, the 30-kDa intermediate, and the 25-kDa mature form (V, Fig. 6A, lane 1 in cells) (Gal et al. 1985). Unglycosylated 36-kDa precursor was detected in both cell extracts and culture medium after a 3-h tunicamycin treatment (V, Fig. 6A, lane 2 in cells and medium). Immunoprecipitation of metabolically labelled cathepsin L showed that, after a 150-min preincubation in the presence of tunicamycin, only the unglycosylated precursor of cathepsin L was synthesized during a 30-min pulse in the presence of the drug (V, Fig. 6B, lane 2). This precursor remained unglycosylated after a 1-h chase in the presence of tunicamycin. It was not processed into the mature form, but secreted into the medium (V, Fig. 6B, lane 5 in cells and medium). The results thus showed that tunicamycin treatment was effective.

Chloroquine treatment (3 h) was shown to accumulate MPRs in large vacuolar endosomes, indicating that it prevented the dissociation of ligands from the receptors (V, Fig. 7A, B) (Brown et al. 1984, Braulke et al. 1987). Moreover, metabolic labelling experiments showed that chloroquine prevented the processing of cathepsin L into the intermediate and mature forms (V, Fig. 6B, lanes 3, 6, 8).

The cells were preincubated with the drugs in a complete culture medium for 3 h to chase newly-synthesized cathepsin L out of the TGN, after which autophagy was induced, in the presence of the drugs, in a serum-free medium for 1 h. Autophagic vacuoles were not observed in Epon sections cut from cells treated with either tunicamycin or chloroquine in a serum-containing culture medium for 3 h. When the treatments were continued for 1 h in a serum-free medium, both early and later autophagic vacuoles were observed. The labelling densities of these autophagic vacuoles for MPR and cathepsin L were determined using double-labelled cryosections. Since only nascent autophagic vacuoles can be identified in cryosections, it is most probable that the counted vacuoles were only formed *after* the 3-h preincubation. MPR and cathepsin L labelling of autophagic vacuoles was similar in both control cells and cells treated with either tunicamycin or chloroquine (V, Table 1; Fig. 8A-C). The results suggest that MPRs do not transport cathepsin L directly from the TGN to autophagic vacuoles. In stead, the enzyme may be transported from the PLC.

6 DISCUSSION

6.1 Structure of the autophagic-vacuole membrane

According to the results of the present (I, II) studies and our previous report (Reunanen et al. 1985), the following model can be proposed for the maturation of the membranes of autophagic vacuoles. Imidazole-buffered OsO_4 staining showed that the membranes of nascent vacuoles contain a lot of unsaturated fatty acids in both Ehrlich ascites cells (I) and mouse hepatocytes (Reunanen et al. 1985). However, the content of these lipids decreases as the vacuoles mature into autolysosomes. According to filipin labelling, the opposite occurs in the cholesterol content of the membranes. The membranes of nascent autophagosomes appear to contain little cholesterol, but the amount of cholesterol rapidly increases, before the inner membrane disappears. The membranes of autolysosomes appear to contain much cholesterol in both Ehrlich ascites cells and mouse hepatocytes, as do the membranes of dense lysosomes in mouse liver. However, the membranes of electron-dense vesicles in Ehrlich ascites cells (putative residual bodies) contain little cholesterol.

Three possible sources can be proposed for the additional cholesterol in the membranes of autophagic vacuoles: (i) the fusion of cholesterol-rich membranes of lysosomal vesicles with the vacuoles; (ii) cholesterol liberated from the segregated organelles by cholesterol-ester hydrolase (Slotte & Ekman 1986); and (iii) the insertion of newly-synthesized cholesterol into the membrane. The first alternative is possible, at least in hepatocytes. In Ehrlich ascites cells, cholesterol is presumably removed from the membranes of residual bodies and reutilized in the cytoplasm.

In Ehrlich ascites cells, the membranes of early (iodoacetate-induced) autophagic vacuoles contain many intramembrane particles, suggesting that the membranes are rich in protein. Some of these membranes also possess attached ribosomes, strongly suggesting that the membranes originate from the rough ER. In mouse hepatocytes, on the other hand, the membranes of early autophagic vacuoles contain little protein. We never observed ribosomes on these membranes. This discrepancy may be due to differences in the putative change of membrane composition during autophagic sequestration (Ericsson 1969b, Dunn 1990a). The partial ATP depletion induced by iodoacetate in Ehrlich ascites cells may also have caused some differences. In both Ehrlich ascites cells and hepatocytes, however, the protein content probably increases as the vacuoles mature into autolysosomes. In hepatocytes, this increase may be due to protein-rich lysosomes fusing with the autophagic vacuoles.

Interestingly, comparable changes in the filipin labelling and density of intramembrane particles have been observed during phagocytosis in Dictyostelium (Favard-Séréno et al. 1981). The filipin labelling and particle density and size increased soon after phagosome closure. This transformation was suggested to be related to the presence of lysosomal enzymes. This implies that the changes are universal and probably have some functional significance.

Cholesterol and unsaturated fatty acids have a significant influence on the fluidity and permeability of membranes, on membrane fusion, and in regulating the activity of membrane-bound enzymes (Chen et al. 1978, Whetton et al. 1983, Presti 1985, Hagve 1988, Minocha et al. 1988, Roerdink et al. 1989). Cholesterol protects membrane phospholipids against hydrolysis by phospholipase A_2 (Fisher et al. 1983). It is possible that high content of unsaturated fatty acids is necessary to produce sufficient fluidity to the membrane during sequestration. Further, the higher cholesterol content in older autophagic vacuoles and lysosomes may protect the limiting membranes of the vacuoles against hydrolysis.

Intramembrane particles have long been associated with membrane proteins (Robertson 1981). Direct evidence has also been presented to support this hypothesis, using an immunocytochemical labelling of membrane particles (Rash et al. 1981) and reconstituted microsomal membranes (Borovyagin et al. 1985). For the proteins in lysosomal membranes, various carrier functions have been proposed (Lloyd & Forster 1986). The lysosomal proton pump (Dell'antone 1988, Moriyama et al. 1984) and amino acid transport system (Bernar et al. 1986) have been characterized. It is possible that the particle density on the membranes of autophagic vacuoles increases with the insertion into the membranes of different carrier proteins, whose function would be e.g. to acidify the contents or transfer the degradation products into the cytoplasm. Lysosomal membrane glycoproteins (lgp120, LIMP I and LIMP V) have been detected in the membranes of autophagic vacuoles (Dunn 1990b).

These may protect the membrane against hydrolytic enzymes. In order to get more specific information on the structure and function of autophagic-vacuole membranes in the future, the membranes should be purified, the lipids analyzed, and the proteins characterized biochemically.

6.2 Vinblastine and autophagy in Ehrlich ascites cells

Vinblastine caused later autophagic vacuoles alone to accumulate in Ehrlich ascites cells. The number of autophagosomes remained at the control level (III). Acid phosphatase activity has also been detected in autophagic vacuoles accumulated during vinblastine treatment (Reunanen & Nykänen 1988). Consequently, in Ehrlich ascites cells, vinblastine does not prevent the entry of lysosomal hydrolases into autophagosomes. However, 0.1 mM vinblastine caused an aggregation of tubuline subunits into paracrystals (I, Fig. 1b). This indicates that the treatment did disrupt the microtubules. The results thus cast doubt on the importance of microtubules in the transport of lysosomal enzymes into autophagosomes in Ehrlich ascites cells.

Since vinblastine induced the accumulation of advanced autophagic vacuoles which contained partially degraded material while not increasing the rate of protein degradation (III), the accumulation must have been caused by retarding degradation in the electron-lucent vacuoles. This suggests that microtubules may be necessary for the delivery of some components obligatory for degradation in autophagic vacuoles.

Leucine and histidine significantly retarded but did not prevent the vinblastine-induced accumulation of electron-lucent vacuoles. The rate of protein degradation was only slightly (non-significantly) lower in the Leu+His and vinblastine-treated cells than in the cells treated only with vinblastine (III). Histidine is presumably metabolized in Ehrlich ascites cells (Doolan & Ward 1987). However, as estimated from the results of these authors, only about 20% of the His could have been metabolized during the 120-min incubation. After this decrease, the concentration of His would still have been about 8 mM, which is about 60 times the concentration in mouse intraperitoneal fluid (Doolan & Ward 1987). In conclusion, since the segregation inhibitors Leu and His could not prevent the accumulation of autophagic vacuoles in the presence of vinblastine, the results suggest that vinblastine also stimulated the formation of new autophagosomes. Similar results were reported by Kovács et al. (1988) and Oliva et al. (1992) for hepatocytes and pancreatic acinar cells.

In the presence of 3-methyladenine, vinblastine did not increase the volume density of autophagic vacuoles. Furthermore, the slight (non-significant) inhibitory effects of 3-methyladenine and vinblastine on

protein degradation were cumulative (III). This is in agreement with results showing that these drugs inhibit different stages in the autophagic degradation pathway: vinblastine inhibits the maturation of autophagic vacuoles, and 3-methyladenine inhibits the formation of new autophagosomes (Seglen & Gordon 1982, 1984). The results of the present study suggest that 3-methyladenine inhibits autophagic sequestration by a mechanism which is more vinblastine-resistant than the inhibitory mechanisms of Leu and His. In accordance with this, Høyvik et al. (1986) found that 3-methyladenine prevented, in isolated hepatocytes, vinblastine-induced [^{14}C]lactose accumulation in autophagic vacuoles.

6.3 MPR, TGN and cathepsin L delivery to autophagic vacuoles in fibroblasts

Many of the early autophagic vacuoles (43-47%) were unlabelled in the cryosections double-labelled for MPR and cathepsin L (IV). These vacuoles can be classified as autophagosomes, i.e., newly-formed vacuoles which have not yet received lysosomal markers. This result supports the view that autophagosomes are formed by a membrane which has no degradative enzymes (Arstila & Trump 1968).

The double labelling of MPR and cathepsin L in the early autophagic vacuoles showed that the occurrence of cathepsin L was dependent on the presence of MPR (IV, Table 1, G-test of independence). In steady state, only slightly more vacuoles were positive for cathepsin L (39-44%) than for MPR (32-36%). These results suggest that considerable amounts of MPR and cathepsin L are delivered simultaneously – perhaps by a common transport route – to autophagosomes. The presence of MPR in the autophagic vacuoles casts doubt on the classical theory that enzymes are delivered to autophagosomes solely by fusion with mature lysosomes, since the lysosomes were essentially devoid of MPRs (IV, Fig. 1d; V, Figs 2B, 5A, B). Tooze et al. (1990) also found MPR and lysosomal enzymes in autophagic vacuoles in guinea pig pancreas. However, in rat liver, MPRs were absent from the majority of autophagic vacuoles (Dunn 1990b). The different results probably reflect differences in the kinetics of MPR recycling during enzyme delivery, or in the role of MPR in enzyme targeting (reviewed by Pfeffer 1988), in different tissues.

To study whether MPRs target enzymes to autophagosomes directly from the TGN, tunicamycin and chloroquine were used (V) to inhibit MPR-mediated transport of newly-synthesized lysosomal enzymes (Imort et al. 1983, Seglen 1983, Nishimura et al. 1988). Tunicamycin and chloroquine did not prevent the delivery of cathepsin L or MPR to early autophagic vacuoles. The results thus suggest that no or only trace amounts of lysosomal enzymes are transported to autophagic vacuoles

by MPRs directly from the TGN. In accordance with our result, Dunn (1990b) and Lawrence & Brown (1992) reported that tunicamycin did not prevent enzyme delivery to autophagic vacuoles in hepatocytes.

6.4 PLC and enzyme delivery to autophagic vacuoles in fibroblasts

Since MPR and cathepsin L were not transported directly from the TGN, the PLC was the most probable source. The fusion of the PLC with autophagic vacuoles was studied with endocytic markers (V).

No endocytic markers (CF or HRP) were detected in autophagic vacuoles after short internalization periods (5-15 min). In these conditions, CF and HRP were found in structures closely resembling early endosomes by morphology, i.e. in small vesicles and tubules in the cell periphery. Several earlier studies have also reported that HRP is localized exclusively in early endosomes after a 15-min uptake (Griffiths et al. 1989, 1990, Ludwig et al. 1991). Thus, our results indicate that early endosomes do not fuse with autophagic vacuoles in rat fibroblasts. In accordance with our results, Tooze et al. (1990) and Gordon et al. (1992) found that early endosomes did not fuse with autophagic vacuoles in guinea pig pancreatic acinar cells and rat hepatocytes, respectively.

The endocytic markers were first detected in autophagic vacuoles after internalization and chase periods long enough to allow transport of the markers to the PLC. Furthermore, the CF or BSA-gold -positive structures observed to fuse with autophagic vacuoles were often morphologically similar to the PLC, i.e., were rich in internal membranes (V, Figs 1A-D; 2A; 3B, C; Griffiths et al. 1988). In addition, both acid phosphatase -positive and negative endosomes (vesicles containing BSA-gold) were observed in fusion profiles with autophagic vacuoles. The PLC has been reported to contain both acid-phosphatase positive and negative regions, whereas lysosomes are all acid phosphatase -positive (Griffiths et al. 1990). Finally, the longer chase periods (which tended to chase more endocytic marker to lysosomes) did not raise the number of marker-positive autophagic vacuoles, whereas the longer internalization periods (which tended to fill the whole PLC in the cells) clearly did so. Continuous uptake of HRP for 1 h has been found to completely fill the PLC of fibroblasts. However, HRP began to significantly fill the lysosomes only after a 2-h uptake (Ludwig et al. 1991). In conclusion, the PLC seems to be the meeting point of the autophagic and endocytic pathways in cultured rat fibroblasts. The PLC has been shown to be capable of dynamic fusion and fission events (Deng et al. 1991). It has earlier been reported as the meeting point of the apical and basolateral endocytic pathways in MDCK cells (Parton et al. 1989) as well as of the phagocytic and

endocytic pathways in macrophages (Rabinowitz et al. 1992).

Even after a 24-h chase, BSA-gold was found in both the PLC and MPR-negative lysosomes. The inability to chase BSA-gold out of the PLC has also been observed by others (Griffiths et al. 1990, Deng et al. 1991). The fluid-phase marker HRP has also been found to exit the PLC very slowly (Ludwig et al. 1991). It is possible that some kind of recycling occurs between the PLC and lysosomes (Deng et al. 1991). Taken together, it is difficult to label lysosomes without concomitant labelling of the PLC. Therefore, our results do not exclude the possibility that also lysosomes may fuse with autophagic vacuoles in fibroblasts.

The present results suggest that the first enzymes and MPRs may be transported to autophagic vacuoles from peripheral parts of the PLC. Firstly, CF (which was mainly detected in large vacuolar parts of the PLC) was not detected in early autophagic vacuoles in cryosections, although low levels of cathepsin L and MPR were present (IV). Only nascent autophagic vacuoles (containing morphologically intact cytoplasm) could be identified in cryosections. Secondly, CF and BSA-gold were observed in autophagic vacuoles containing partially degraded cytoplasm, and they appeared to enter in large, complex structures (V). By contrast, HRP was also found in early autophagic vacuoles containing morphologically intact cytoplasm (V). Figures such as 4D-F in V suggest that HRP may be transported in small vesicles or tubules (50-150 nm in diameter). These vesicular-tubular profiles most likely belong to the PLC which consists of reticular and vacuolar regions (Griffiths et al. 1988). Therefore, it is possible that the first lysosomal enzymes and MPRs are transported to autophagic vacuoles in small PLC-derived vesicles or tubules which are reached by HRP but not by the less sensitive markers CF and BSA-gold. Later autophagic vacuoles may arise by fusion with the larger vacuolar parts of the PLC). This fusion would form more complex structures containing internal membranes. The final degradation of the cytoplasmic material may occur in these complex structures, or the material may be transported to lysosomes for degradation.

Tooze et al. (1990) reported that in exocrine pancreas early autophagic vacuoles first received endocytosed HRP which was proposed to originate from the endocytic route immediately after early endosomes. The vacuoles then received lysosomal cathepsins and MPR. Gordon et al. (1992) reported that in isolated rat hepatocytes, the fluid-phase endocytic markers entered autophagic vacuoles before the degradation of cytoplasmic material began. Taken together, the fluid-phase endocytic route seems to fuse with autophagic vacuoles before the bulk of lysosomal enzymes is delivered from the PLC (V, Tooze et al. 1990). In hepatocytes, in contrast, the bulk of lysosomal enzymes seems to be delivered by fusion with mature lysosomes (Lawrence & Brown 1992). However, the first proteolytic enzymes and MPRs are probably delivered to autophagic vacuoles earlier in fibroblasts (IV, V) than in exocrine pancreas and liver.

6.5 Acidification and cathepsin L delivery to autophagic vacuoles in fibroblasts

In early autophagic vacuoles, the occurrence of cathepsin L and MPR were dependent on the presence of DAMP (IV, Table 1, G-test of independence). Furthermore, the proportions of vacuoles positive for cathepsin L (39-44%) and DAMP (42%) were equal. This suggests that in fibroblasts, acidification begins simultaneously with the delivery of the enzyme. In guinea pig pancreas, DAMP accumulation began and acid phosphatase activity appeared prior to the delivery of cathepsins D and B (Tooze et al. 1990). And further, in rat liver, DAMP accumulation was extensive before the delivery of cathepsin L (Dunn 1990b).

In the present study (IV), the inhibition of acidification by monensin treatment was not able to prevent the delivery of cathepsin L or MPR to autophagosomes. Monensin at a concentration of 10 μM has been shown to raise the pH of acidic organelles in one minute (Yamashiro & Maxfield 1987). Therefore, since autophagy was induced in the presence of the drug, it is unlikely that any enzymes could have been delivered to early autophagic vacuoles before the drug had raised the pH. The result thus suggests that acidification is not an obligatory step before the delivery of lysosomal enzymes to autophagosomes in fibroblasts. FIGURE 3 presents a summary of the autophagic pathway in fibroblasts.

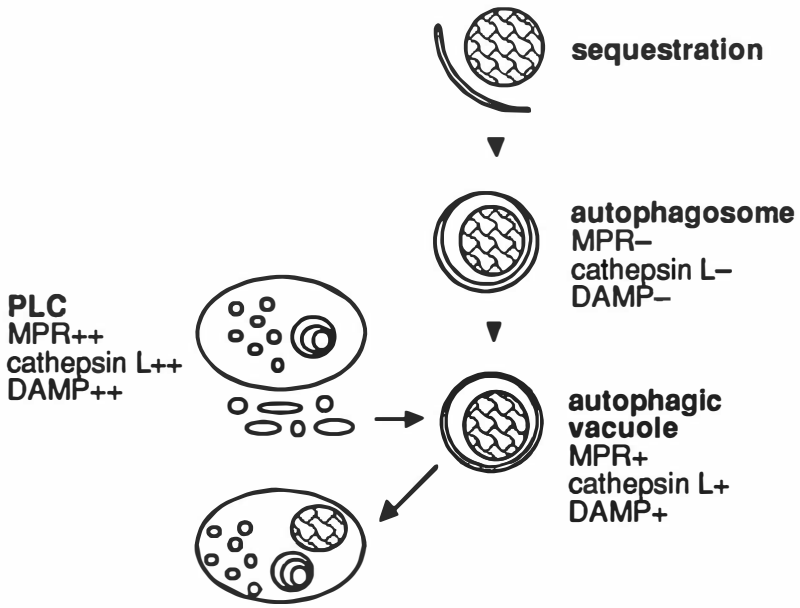

FIGURE 3. Schematic summary of autophagy in rat fibroblasts. Autophagosomes are formed by membranes which contain no lysosomal enzymes. The first lysosomal enzymes and proton pumps may be delivered from the reticular parts of the PLC. Later, the autophagic vacuoles deliver their contents to vacuolar parts of the PLC. The term 'autolysosome' is omitted since 'lysosomes' are defined to be MPR-negative (Kornfeld & Mellman 1989). The cytoplasmic material may be degraded in the PLC, or be transported to lysosomes for degradation.

7 CONCLUSIONS

The maturation of autophagic vacuoles was studied with electron microscopical methods in Ehrlich ascites cells, mouse hepatocytes and rat fibroblasts. The results show the following.

1. In Ehrlich ascites cells and mouse hepatocytes, the membranes of nascent autophagic vacuoles contained little cholesterol, whereas the membranes of older, double- and single-membrane limited vacuoles were cholesterol-rich (I, II). The membranes of mouse-liver lysosomes were also rich in cholesterol, whereas those of residual bodies in Ehrlich ascites cells were not. The additional cholesterol in the membranes of later autophagic vacuoles may be derived from cholesterol-rich lysosomes or other vesicles fusing with the vacuoles.

2. The density of membrane proteins (intramembrane particles) on the membranes of nascent autophagic vacuoles was high in Ehrlich ascites cells and low in mouse hepatocytes (I, II). In both cell types, however, the density was higher in later vacuoles. Mouse-liver lysosomes were very protein-rich. The increase in particle density in autophagic vacuoles may be associated with the delivery of lysosomal enzymes, and may also be due to the insertion of structural proteins and proton pumps into the membrane.

3. In Ehrlich ascites cells, disruption of microtubules by vinblastine caused an accumulation of later autophagic vacuoles (III). Hence, vinblastine did not prevent the entry of lysosomal enzymes into autophagosomes. Thus, the accumulation must have been caused by retarded degradation in the later vacuoles. Since the segregation inhibitors Leu

and His could not prevent the vinblastine-induced accumulation of autophagic vacuoles, vinblastine also stimulated the formation of new autophagosomes. However, 3-methyladenine inhibited autophagic segregation by a vinblastine-resistant mechanism.

4. Cathepsin L was delivered together with MPR into autophagosomes in rat fibroblasts (IV). Inhibitor studies with tunicamycin and chloroquine showed that cathepsin L was not transported by MPRs directly from the trans-Golgi network (V). Experiments with endocytic markers showed that autophagic vacuoles fused with the PLC (V). The results thus suggest that cathepsin L and MPR are transported to autophagosomes from the PLC.

5. Autophagic vacuoles were acidified simultaneously as they acquired cathepsin L and MPR in fibroblasts (IV). Inhibition of acidification did not prevent the delivery of cathepsin L. This suggests that the enzyme and proton pump both originate from the PLC, and that acidification is not obligatory for enzyme delivery.

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Eeva-Liisa Punnonen

Yhteenveto

Hienorakennetutkimus solujen autofagosytoosista. Kalvojen rakenne ja entsyymikuljetuksen reitti

Pääosa solun organellien ja sytoplasman rakenneosien hajotuksesta tapahtuu autofagosytoosin välityksellä. Autofagisen hajotuksen nopeus kuuluu tekijöihin, joilla solut säätelevät kasvunopeuttaan. Autofagisen vakuolin muodostuessa litistynyt kalvopussi kiertyy rakkulaksi ja sulkee sisäänsä osan sytoplasmaa. Näin muodostuu kaksoiskalvon ympäröimä rakkula. Tähän rakkulaan kuljetetaan lysosomaalisia hajottavia entsyymejä, ja sen sisältö hajoaa pieniksi molekyyleiksi, jotka voidaan kuljettaa takaisin sytoplasmaan. Tässä tutkimuksessa selvitettiin autofagisten vakuolien kypsymiseen liittyviä tapahtumia Ehrlich ascites-soluissa, hiiren maksassa sekä viljellyissä rotan fibroblasteissa. Tärkeimmät tulokset olivat seuraavat.

1. Vastamuodostuneiden autofagisten vakuolien kalvot sisälsivät vähän kolesterolia sekä Ehrlich ascites -soluissa että hiiren maksasoluissa. Vakuolien kypsyessä kalvojen kolesterolipitoisuus kuitenkin kasvoi. Hiiren maksasolujen lysosomien kalvoissa oli myös paljon kolesterolia. Autofagisten vakuolien kalvot voivat saada kolesterolia vakuoleihin yhtyvien lysosomaalisten vesikkeleiden kalvoista.

2. Autofagisten vakuolien kalvoissa oli paljon proteiineja (kalvopartikkeleita) Ehrlich ascites -soluissa, kun taas hiiren maksasolujen vakuolien kalvoissa oli hyvin vähän kalvopartikkeleita. Molemmissa solutyypeissä proteiinien määrä kuitenkin kasvoi vakuolien kypsyessä. Hiiren maksan lysosomien kalvoissa oli myös paljon proteiineja. Autofagisten vakuolien kalvojen proteiinilisäys saattaa liittyä lysosomaalisten entsyymien kuljetukseen: kalvoihin kuljetetaan lysosomaalisia kalvoproteiineja ja protonipumppuja.

3. Mikrotubulusten hajottaminen vinblastiinilla aiheutti osittain hajonnutta sytoplasman materiaalia sisältävien autofagisten vakuolien kerääntymisen Ehrlich ascites -soluihin. Varhaisvaiheisten vakuolien määrä ei lisääntynyt. Tämä osoittaa, että mikrotubulusten puuttuminen ei estänyt lysosomaalisten entsyymien kuljetusta vakuoleihin. Vakuolien kerääntyminen johtui ilmeisesti entsyymien toiminnan viivästymisestä; mahdollisesti jonkin hajotuksessa tarvittavan tekijän kuljetus estyi. Leusiini ja histidiini (jotka estävät autofagisten vakuolien muodostumisen) eivät kyenneet estämään vinblastiinin aiheuttamaa vakuolien

kertymistä. Tämä viittaa siihen, että vinblastiini myös indusoi uusien vakuolien muodostumista. 3-metyyliadeniini kuitenkin esti uusien vakuolien muodostumisen myös vinblastiinin läsnäollessa.

4. Huomattava osa katepsiini L:stä (lysosomaalinen entsyymi) kuljetettiin autofagisiin vakuoleihin samanaikaisesti mannoosi-6-fosfaattireseptorin (MPR) kanssa rotan fibroblasteissa. Inhibiittorikokeet tunikamysiinillä ja klorokiinilla kuitenkin osoittivat, että katepsiini L:ää ei kuljetettu suoraan trans-Golgista autofagisiin vakuoleihin. Endosytoosimerkkiaineiden avulla voitiin osoittaa, että autofagiset vakuolit yhtyivät myöhäisten endosomien kanssa. Tulokset viittaavat siihen, että suuri osa katepsiini L:stä kuljetetaan autofagisiin vakuoleihin myöhäisistä endosomeista.

5. Autofagiset vakuolit muuttuivat happamiksi samanaikaisesti kun niihin kuljetettiin katepsiini L ja MPR rotan fibroblasteissa. Entsyymin kuljetus ei estynyt vaikka pH:n lasku estettiin monensiinilla. Tämä viittaa siihen, että sekä entsyymi että protonipumppu tulevat myöhäisistä endosomeista ja että pH:n lasku ei ole entsyymin kuljetuksen edellytys.

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ORIGINAL PAPERS

I

Filipin labelling and intramembrane particles on the membranes of early and later autophagic vacuoles in Ehrlich ascites cells

by

Eeva-Liisa Punnonen, Hilkka Reunanen, Pirkko Hirsimäki
and Kari Lounatmaa

Virchows Archiv B Cell Pathology 54: 317-326, 1988

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II

Intramembrane particles and filipin labelling on the membranes of autophagic vacuoles and lysosomes in mouse liver

by

Eeva-Liisa Punnonen, Kaarina Pihakaski, Kari Mattila, Kari Lounatmaa and Pirkko Hirsimäki

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III

Effects of vinblastine, leucine and histidine, and 3-methyladenine on autophagy in Ehrlich ascites cells

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IV

Autophagy, cathepsin L transport, and acidification in cultured rat fibroblasts

by

Eeva-Liisa Punnonen, Soile Autio, Varpu S. Marjomäki
and Hilkka Reunanen

Journal of Histochemistry and Cytochemistry 40: 1579-1587, 1992

https://doi.org/10.1177/40.10.1326577

V

Autophagic vacuoles fuse with the prelysosomal compartment in cultured rat fibroblasts

by

Eeva-Liisa Punnonen, Soile Autio, Helena Kaija and Hilkka Reunanen

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