REVIEW ARTICLE

Physiological functions of endosomal proteolysis

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INTRODUCTION

Endocytosis is a mechanism used by cells for uptake of macromolecules from extracellular fluid. The uptake may take place in specialized regions of the plasma membrane termed clathrin-coated pits, but uptake outside these regions has been reported [1]. Macromolecules may be bound to receptors before uptake or may be present in the fluid phase of the forming endocytic vesicle. The proportion of fluid taken up through the two pathways depends on the cell type. In hepatocytes, the bulk of fluid-phase endocytosis seems to take place outside clathrin-coated pits [2]. Macrophages and other cells can be stimulated by growth factors to increased fluid-phase endocytosis via so-called macropinosomes [3–5]. After formation of clathrin-coated vesicles, the coat is shed and uncoated ‘primary endosomes’ are formed [6]. These are small vesicles (100 nm diam.) that transport their cargo to early endosomes (Figure 1). Clathrin-independent endocytosis also leads to formation of primary endosomes that may be even smaller than those resulting from clathrin-dependent endocytosis [7]. Material delivered by one of these pathways may take one of four possible routes once in the early endosomes [6]: (1) the receptor–ligand complex may be directed to lysosomes for degradation; (2) the ligand may dissociate from the receptor and

follow the pathway to the lysosome whereas the receptor is recycled back to the plasma membrane; (3) the receptor–ligand complex may (in epithelial cells) be transported to the opposite side of the cell; (4) the receptor–ligand complex may be recycled back to the plasma membrane (retroendocytosis). In addition it is likely that all receptor–ligand complexes to some extent are recycled back to the plasma membrane.

The mechanism whereby the ligand and/or the receptor is transported to the lysosomes is not known. Two main models have been proposed; they have been termed the ‘maturation model’ [8,9] and the ‘vesicle shuttle model’ [10]. The maturation model proposes that the various organelles involved in endocytosis are formed by maturation of the primary endosomes (formed by internalization of the plasma membrane). Recycling components would leave the endosomes by vesicles budding off and new components such as lysosomal enzymes and lysosomal glycoproteins would enter by vesicles fusing with the maturing endosomes. The vesicle shuttle model proposes a series of stable organelles (endosomes, lysosomes) that communicate through shuttling vesicles. The transport vesicles in this model include the primary endosomes and the endocytic carrier vesicles that are assumed to be budding off from the early endosomes. Transport vesicles operating between late endosomes and lysosomes have not yet been described. Selected regions of the late endosomes may bud off and carry their cargo to the terminal lysosomes. It is clear that only a selected portion of the late endosome is transported to lysosomes. The mannose-6-phosphate receptors (M6PRs) are not found in the lysosomes but are present in the prelysosomal compartment [11]. The M6PRs recycle between the endosomes and the *trans*-Golgi compartment (TGN). If a maturation model were valid one would expect that the M6PRs were introduced into the maturing endosome by fusion with Golgi-derived vesicles and subsequently, prior to the formation of lysosomes, the M6PRs would be retrieved by vesicles budding from late prelysosomal organelles. It is likely, in liver cells, that the endosomes are transported to a pre-existing lysosome rather than maturing into a lysosome, since in cell-free systems reconstitution of endosome–lysosome fusion requires the presence of lysosomes for the processing of ligand originally loaded in endosomes [12]. A third model for transport along the endocytic pathway has been postulated by Hopkins et al. [13]. They found that endocytosed markers are transported along continuous reticular tubules centripetally from the periphery of the cells. As discussed in the following text, different experimental systems may support one of these models, but often without excluding the others. It is therefore likely that the endocytic pathway contains elements of all three models, depending on cell type, state of activation, etc.

Multivesicular bodies (MVBs) are endocytic vesicles that occur to a variable extent in most cells. They are spherical vacuoles, 200–800 nm in diameter, containing small (40–80 nm diam.) vesicles [14]. In hepatocytes early endosomes (CURL) seem to

Figure 1 Endocytic transport pathways in a non-polarized cell

Primary endocytic vesicles are invaginated from clathrin-coated (CCV) (1) and non-coated regions (NCV) (2) of the plasma membrane. In early endosomes (EE), receptors are sorted from ligands and returned to the plasma membrane (3) via recycling vesicles (RV). Ligands destined for degradation are brought in transport vesicles (TV) (4) along microtubuli (MT) to late endosomes (LE). Newly synthesized lysosomal enzymes are introduced from TGN into the endocytic pathway either via the plasma membrane or directly to late endosomes (6). From late endosomes, enzymes and their substrates are transferred to lysosomes by vesicular transport or maturation (5).

Abbreviations used: CIM6PR, cation-independent mannose-6-phosphate receptor; CPL, compartment for peptide loading; DAB, diaminobenzidine; EGF, epidermal growth factor; ER, endoplasmic reticulum; HRP, horseradish peroxidase; IDE, insulin-degrading enzyme; IGF-I and -II, insulin-like growth factor I and II; LDL, low-density lipoprotein; MHC, major histocompatibility complex; M6PR, mannose-6-phosphate receptor; MVB, multivesicular body; NEM, *N*-ethylmaleimide; PTH, parathyroid hormone; TC, tyramine cellobiose; TGN, *trans*-Golgi compartment; VLDL, very-low-density lipoprotein; VTG, vitellogenin.

develop into MVBs [15,16]. In the human carcinoma cell line HEP-2 the MVB is the dominating endocytic organelle [14]. The internal vesicles are probably formed by inward budding of the outer MVB membrane. Following fusion with lysosomes, or alternatively after receiving lysosomal enzymes from the TGN, the internal vesicles will be degraded. It has been shown that epidermal growth factor (EGF) receptors are completely degraded in lysosomes of liver cells [17]. This is compatible with their sequestration in internal vesicles of MVBs and subsequent degradation in lysosomes. Some controversies about the endocytic process may simply be due to a considerable variation in the kinetics of the process and the morphology of the organelles involved. All models of endocytosis seem to operate with a common set of organelles: primary endosomes, formed from clathrin-coated or uncoated regions of the plasma membrane, bring ligand-receptor complexes to early endosomes that consist of tubular and luminal parts. The next organelle in the pathway to lysosomes is multivesicular to varying extents: this organelle may in some cells serve as a carrier vesicle to a system of prelysosomal organelles [18] and in other cells mature into late MVBs that gradually become lysosomes [14].

It was previously thought that degradation of endocytosed macromolecules took place exclusively in terminal lysosomes. Recent reports have demonstrated, however, that partial or complete hydrolysis of lysosomal enzyme substrates may take place in prelysosomal components of the endocytic pathway. Therefore, intracellular degradation of endocytosed substrates seems to take place in a 'digestive tract' and not only in the terminal lysosomes. The purpose of the present review is to discuss the occurrence and function of endosomal proteolysis.

PROTEOLYTIC MODIFICATIONS OF LYSOSOMAL ENZYMES IN THE ENDOSOMES

Hydrolysis of substrates in endosomes requires hydrolases and a suitable pH for the enzymes. Both these conditions may be met. The M6PRs transport lysosomal enzymes to a prelysosomal compartment [19]. The enzymes dissociate from the receptors because of the low pH and the M6PRs recycle to the TGN whereas the phosphorylated enzymes are brought to the terminal lysosomes. Lysosomal enzymes may also (by default) be carried to the plasma membrane in association with the cation-independent M6PR (CIM6PR) [20]. The receptor contains signal in its cytoplasmic tail for internalization via clathrin-coated pits [21] and the enzymes may therefore be introduced into the endocytic pathway even at the level of the formation of endocytic vesicles. Ludwig and co-workers [22] showed that newly synthesized CIM6PRs were introduced into the endocytic pathway at the level of early endosomes; as much as 40% of the receptors were found in these organelles. Available data thus demonstrate that lysosomal hydrolases are present all along the endocytic pathway. The pH of the endosomes has been shown to decrease along the endocytic pathway [23–25] but could be sufficiently low even in early endosomes to allow degradation of some substrates.

In addition to removal of the signal peptide in the endoplasmic reticulum (ER), the newly synthesized lysosomal enzymes may be subject to additional processing. Several lysosomal proteases are synthesized as inactive zymogens and require proteolytic processing to attain full activity [20,26]. Examples of proteases that require proteolytic cleavage (fragmentation) for activation include cathepsin C [27–30], cathepsin D [31–33], cathepsin H [34], cathepsin B [35,36] and cathepsin L [37–39]. Cathepsin D probably activates itself [31] and may even activate other cathepsins such as cathepsin L and cathepsin B [37,40].

Whereas the biochemistry of fragmentation and exoproteolytic maturation of lysosomal enzymes have been clarified in great detail [20], less is known about the intracellular sites at which the proteolytic processing takes place. It is reasonable to assume that most of the proteolytic processing takes place in lysosomes. However, it has become increasingly clear that proteolysis also takes place in prelysosomal compartments and this requires activation of the relevant proteases. Several studies have shown that activation of cathepsin D takes place in a prelysosomal compartment and that this enzyme also catalyses endosomal proteolysis. Gieselmann et al. [41] studied biosynthesis and transport of cathepsin D in cultured human fibroblasts. The maturation kinetics for cathepsin D were followed by subcellular fractionation and immunoprecipitation of cells labelled with [³⁵S]methionine in Percoll-density gradients. It was found that after a pulse of 20 min and a 30 min chase most of the cathepsin D was present in light fractions of the gradient as 53 kDa precursors, but about 22% of the label was associated with a 47 kDa intermediate. The results suggest that the processing of the 53 kDa precursor to the 47 kDa intermediate occurs mainly in the low-density organelles, whereas the conversion of the 47 kDa intermediate into the mature 31 kDa form takes place predominantly in heavy lysosomes.

The notion that proteolytic processing of the cathepsin D proenzyme may take place in endosomes was supported by a later study by Rijnboutt et al. [42]. In this study endosomes were labelled with horseradish peroxidase (HRP) conjugated to transferrin. This complex is recycled following endocytic uptake and will therefore label the endosomes and not lysosomes. To label the entire endocytic pathway the cells were allowed to take up HRP which is a marker of the luminal content in the endosomes. If cells are incubated in the presence of diaminobenzidine (DAB) and H₂O₂, DAB polymers will form in the HRP-containing vesicles resulting in a detergent-insoluble protein precipitate [43,44]. Co-localization of cathepsin D with HRP can be visualized by loss of detectable cathepsin D. The results showed that the formation of the cathepsin D intermediate (44 kDa) took place in compartments accessible to endocytosed HRP as well as transferrin-HRP. Transferrin-HRP did not at any time co-localize with the mature 31 kDa species. The final processing therefore seems to take place in lysosomes.

PRELYSOSOMAL DEGRADATION OF ENDOCYTOSED LIGANDS

The presence of active proteinases and other lysosomal enzymes in endosomes makes possible the hydrolysis of endocytosed and endogenous substrates in these organelles if pH and ionic conditions are suitable. A hint of the role of endosomal proteolysis may be obtained by determining the kinetics of the degradation of endocytosed proteins and at the same time following their intracellular transport by means of subcellular fractionation or by microscopy techniques. This approach has been used to follow degradation of endocytosed molecules along the endocytic pathway particularly in liver cells and in macrophages.

Endosomal hydrolysis in liver cells

Suggestive evidence of prelysosomal degradation of asialo-orosomucoid in isolated rat hepatocytes was obtained using ligand labelled with [¹²⁵I]-tyramine cellobiose (TC). The labelled degradation products, [¹²⁵I]-TC attached to small peptides, do not leak out of the endocytic vesicles and may therefore serve as markers for the organelles in which degradation really takes place [45–47]. It was found that degradation (formation of acid-soluble radioactivity) was initiated in vesicles of lower density

than the bulk of lysosomes [45,46]. Subsequently, the labelled degradation products were found in the high-density lysosomes. The simplest interpretation of these data is that degradation is initiated in endosomes and that the degradation products as well as undegraded ligand were subsequently transferred to lysosomes where degradation was completed.

Wattiaux and co-workers also employed ligands labelled with ^{125}I -TC in combination with subcellular fractionation (in sucrose- and Percoll-density gradients) to study intracellular transport and degradation of endocytosed glycoproteins in liver endothelial cells *in situ* [48–50]. To differentiate between endosomal and lysosomal localization of the endocytosed ligand, density shift of lysosomes was induced either by Triton WR-1339 [48] or by means of invertase [51–53]. Triton WR-1339 reduces the density of hepatic lysosomes in general [49,54] whereas yeast invertase increases selectively the density of lysosomes in liver endothelial cells [55–58]. To determine whether a ligand had entered a lysosomal compartment, homogenates were incubated with glycylphenylalanyl 2-naphthylamide which selectively induces osmotic lysis of lysosomes [51,59]. It was found that partial proteolysis of ^{125}I -TC-labelled formaldehyde-treated albumin starts within 1 min of initiation of uptake. This degradation took place in organelles that had not been affected by the density perturbants. The first cleavage of the endocytosed ligand was inhibited by pepstatin A, an inhibitor of cathepsin D, but not by leupeptin, a thiol proteinase inhibitor. It was therefore suggested that cathepsin D may play an important role in the initiation of endosomal proteolysis [60]. A similar prominent role for cathepsin D in endosomal proteolysis has been observed in macrophages (see below). Casciola-Rosen and Hubbard [61] used perfused rat liver to investigate the role of endosomes versus lysosomes in the hydrolysis of endocytosed material. It was assumed that endosomal degradation should take place in cells at 16 °C and that it should be detectable relatively rapidly (<15 min) after initiation of uptake at 37 °C. It has been shown that transport from endosomes to lysosomes is arrested at 16 °C in rat liver [62]. The liver cells were allowed to endocytose small fluorogenic substrates from which fluorescent products are generated by a single cleavage [61]. Using such substrates hydrolysis catalysed by acid phosphatase and aryl sulphatase was clearly seen in endosomes whereas no β -hexosaminidase activity was detected prelysosomally. However, when proteolysis was measured as acid-soluble radioactivity, no endosomal degradation of ^{125}I -labelled galactosylated albumin or ^{125}I -mannosylated albumin was observed. This is in contrast to results obtained with isolated rat liver parenchymal cells [45,46,63]. The kinetics of intracellular transport and degradation in hepatocytes may differ from that seen in the cells *in situ*.

Prelysosomal hydrolysis of lipoproteins in the liver

The liver plays a central role in lipoprotein metabolism. The liver expresses receptors that mediate endocytic uptake of, for example, low-density lipoprotein (LDL), chylomicron remnants, β very-low-density lipoprotein (β -VLDL) as well as modified (oxidized) LDL [64–66]. Runquist and Havel [67] analysed lysosomal enzymes in purified endosomal fractions from rat liver [68] and found high activity of both acid phosphatase and cholesteryl ester hydrolase. Considerably lower activities were found of cathepsin D, β -acetylglucosaminidase and aryl sulphatase. In purified endosomal fractions containing ^{125}I -labelled LDL, partial proteolysis of the apoB moiety took place at pH 4.5. The protease responsible for the proteolysis was likely to be cathepsin D since degradation was inhibited by pepstatin. This observation is in agreement with other reports indicating

that cathepsin D plays a pivotal role in endosomal proteolysis [69]. Hydrolysis of endocytosed LDL cholesteryl esters *in vitro* was also demonstrated. The functional significance of the latter finding is uncertain, since very little degradation of both ^{125}I -labelled apoB or [^3H]cholesteryl linoleate was seen in endosomal fractions isolated from animals 15 min after injection of the labelled lipoprotein. The pattern of enzyme activities in the endosomal fractions is not reflected in a corresponding hydrolysis of endocytosed substrates. Thus cholesteryl esters do not seem to be hydrolysed in endosomes although the activity of cholesteryl ester hydrolase is high. On the other hand, aryl sulphatase-specific substrates are degraded in endosomes [61] although the activity of aryl sulphatase in the endosomal fractions is low [70]. The enzyme activities in the isolated endosomal fractions may not, however, reflect the *in vivo* situation because the recovery of purified organelles was low and the animals were treated with 17α -oestradiol. Furthermore, part of the enzyme activities in the endosomal fractions may originate in non-parenchymal liver cells. The non-parenchymal liver cells and particularly the liver endothelial cells contain high levels of aryl sulphatase and cathepsin D [71–75]. Hornick et al. [76] used a purification procedure similar to that employed by Runquist and Havel [67] to prepare endosomal fractions. They found that isolated endosomes could hydrolyse VLDL triacylglycerol following its endocytic uptake in hepatic endosomes. Evidently, more work is needed to clarify the role of endosomes in the hydrolysis of both triacylglycerol and cholesteryl esters.

The chylomicrons also contain, in addition to cholesteryl ester, retinyl esters, and following hydrolysis and delivery of triacylglycerol to extrahepatic tissues, the chylomicron remnants containing retinyl esters are removed from the circulation by endocytosis in the rat liver parenchymal cells [77,78]. The chylomicron remnants with their retinyl esters are hydrolysed and the retinol is subsequently carried to the ER [79,80]. In the ER the retinol is associated with retinol-binding protein and secreted [79,80]. The site of retinyl ester hydrolysis is not known. However, work presented by Blomhoff et al. [81] and by Blaner and co-workers [82] clearly showed that the retinyl ester hydrolysis is a prelysosomal event. The subcellular location of retinyl ester hydrolase may be the early endosome.

Endosomal proteolysis in macrophages

The very complicated endocytic pathways of macrophages make it difficult to pinpoint the site of degradation of endocytosed proteins. It has been shown, for instance, that a considerable proportion (> 30 %) of phagocytosed bacteria [83,84] were in phagosomes that had fused with tubular lysosomes within 10 min. Similar kinetics were noticed by Mayorga et al. [85] who found that fusion of phagosomes with lysosomes was initiated 3 min after the beginning of phagocytic uptake of *Staphylococcus aureus*. These results indicate that lysosomes may be involved very early in the endocytic process.

There is, nevertheless, evidence that prelysosomal proteolysis may take place both in early endosomes and in newly formed phagosomes. Stahl and co-workers have, in a series of studies, [69,86–91] presented data indicating that endosomal proteolysis of BSA [86], peptide hormones [90] and protein toxins [88] may take place in macrophages. Diment and Stahl [86] showed that degradation of mannosylated albumin started within 6 min of initiating uptake in alveolar macrophages. The labelled ligand was at this point in a light organelle which could be separated from the secondary lysosomes on Percoll-density gradients. The degradation of ^{125}I -labelled mannosylated albumin was inhibited by the cathepsin D inhibitor pepstatin A, and cathepsin D was

shown by immunoprecipitation analysis of gradient fractions to be present in endosomes [69]. The enzyme had a subunit molecular mass of 46 kDa and was partially membrane-bound. The mature cathepsin D (molecular mass 31 kDa) is probably formed from the 46 kDa precursor in the lysosomes [41].

Endosomal proteolysis is evidently limited to certain proteins that are susceptible to proteolysis at relatively high pH (pH 6). For instance, if an immune complex consisting of IgG and dinitrophenylated BSA is taken up in macrophages then the antigen (BSA) is rapidly degraded in endosomes whereas the antibody (IgG) is resistant to endosomal degradation and will therefore be transported to secondary lysosomes before degradation commences [91].

Endosomal proteolysis in macrophages can be reconstituted in a cell-free system [85,87,88]. The process requires ATP, which is evidently necessary for endosomal acidification. Mayorga et al. [85] were even able to reconstitute the transport of ligands from plasma-membrane-derived vesicles to proteolytic endosomes *in vitro*. This fusion event was dependent on cytosolic *N*-ethylmaleimide (NEM)-sensitive factors and potassium. The luminal pH of endosomes may decrease from near neutral to acidic values following formation of these organelles. The fluctuation in endosomal pH may allow proteolysis to take place at different pH values. Blum et al. [88] showed that degradation of ricin A chain, which is internalized via the mannose receptor in macrophages, had already started after 2–3 min. Proteolysis of ricin A chain in isolated endosomal vesicles took place even at neutral pH. Both cathepsin D and cathepsin B seemed to be involved in degradation of the toxin. Both enzymes are active at low pH, but the cysteine proteinase cathepsin B catalysed toxin proteolysis even at neutral pH [92].

ENDOSOMAL PROTEOLYSIS OF PEPTIDE HORMONES

Peptide hormones are taken up in target cells by receptor-mediated endocytosis and are subsequently processed in the endocytic pathway. Four types of processing have been observed: (a) EGF is partially degraded in prelysosomal compartments and then completely degraded in lysosomes [93–99]; (b) parathyroid hormone (PTH) is activated in macrophages by partial proteolysis in endosomes, and subsequently released to act on target cells [90,100–102]; (c) insulin and glucagon are, at least in some target cells, completely degraded in endosomes [103–111]; (d) some peptide hormones may be degraded in lysosomes (Table 1). The physiological role of endosomal proteolysis of hormones is not fully understood. PTH is activated by partial proteolysis in the endosomes and the activated hormone is subsequently released to act on target cells. It remains to be seen whether other signal molecules are treated in a similar way. It has been suggested that endosomes may play a role in facilitating the transmembrane signalling for insulin and growth factors whose receptors possess tyrosine kinase activity. Active receptor kinase in endosomes has been demonstrated for insulin [112] and EGF [113] and it has even been suggested that insulin-receptor internalization is

required for insulin-dependent processes [114]. Endosomal degradation of insulin, glucagon and growth factors may therefore be needed to effectively terminate the signal.

Receptor-mediated endocytosis and degradation of insulin and glucagon

Insulin binds to a receptor which contains a tyrosine-specific protein kinase [115]. Activation of the tyrosyl kinase is thought to play a central role in transmission of the regulatory signals, and autophosphorylation of the receptor appears to be required for endocytosis. The cytoplasmic tail of the insulin receptor contains a signal that brings the ligand–receptor complex to coated pits where internalization takes place [114]. The signal consists of the amino acid sequence NPXY (where X stands for any amino acid) and is common for several intrinsic membrane proteins such as the LDL receptor, the insulin receptor and the insulin-like growth factor I (IGF-I) and EGF receptors [116]. The binding of insulin to its receptor stimulates internalization of the receptor–ligand complex with subsequent degradation of insulin and recycling of the receptor [117]. A number of studies have concluded that endosomes are a major site of degradation for insulin [103–105,107,109,114,118–121]. This conclusion is based on the findings that degradation of insulin starts too early after uptake to be accounted for by lysosomes [105,114] and that insulin degradation can be demonstrated in endosomal fractions [103,104,121]. The prelysosomal processing of insulin can be clearly appreciated when the kinetics of insulin processing is compared with that of a ligand that is known to be degraded in lysosomes. This was done by Backer and co-workers [105] who compared the intracellular processing of insulin with that of the insulin-like growth factor II (IGF-II) in the rat hepatoma cell line Tao. The IGF-II receptor (identical to the CIM6PR) has been studied extensively, and biochemical and morphological data show that this receptor mediates transport to a late endosomal/prelysosomal compartment that either matures or delivers its contents to a terminal lysosomal compartment [122–124]. It was found that insulin was degraded within 4–5 min after internalization whereas a lag of 20–30 min occurred between internalization of IGF-II and the appearance of degradation. These data are compatible with lysosomal degradation of IGF-II, whereas insulin degradation must start prelysosomally, in endosomes.

An endosomal site of degradation of insulin is furthermore compatible with other observations: ATP-dependent acidification is required for insulin degradation both in isolated endosomes [103,104,121,125] and in intact cells. Thus, degradation is inhibited by ionophores such as nigericin and monensin, by proton pump inhibitors such as NEM, DCCD, vanadate and amiloride, and by weak bases (chloroquine) [104,125–128]. Insulin seemed to be selectively degraded in endosomes, since other ligands present in the same early endosomes (asialo-orosomucoid, EGF and prolactin [104,129,130]) were not degraded under conditions that lead to complete degradation of insulin. Hamel et

Table 1 Endosomal processing of peptide hormones

Hormone	Receptor signal	Processing	Function of processing	Receptor recycling	Reference
Insulin	Tyr-kinase	Complete	Signal termination	Yes	[114]
Glucagon	Adenylate cyclase	Complete	Signal termination	Yes	[125]
EGF	Tyr-kinase	Partial	Signal termination	No	[15]
PTH	Adenylate cyclase	Partial	Hormone activation	Yes	[100]

al. [103] demonstrated insulin fragments in endosomes that corresponded to the *in vivo* sites of hormone hydrolysis and it has been shown that endosomes *in vitro* generate insulin fragments cleaved at the same sites as observed *in vivo* [103,104,120,121]. Pease et al. [107] calculated that the rate of insulin degradation in hepatic endosomes is consistent with the rate of insulin clearance *in vivo*.

Although insulin seems to be degraded as a consequence of receptor-mediated endocytosis, the specific enzymes involved in insulin degradation are still not entirely clear. Several observations including kinetic characteristics, the effect of inhibitors, and the effect of monoclonal and polyclonal antibodies have supported the notion that the so-called 'insulin-degrading enzyme' (IDE) may be the primary cell insulin-degrading enzyme [106,131–134]. It has, however, been particularly difficult to reconcile intra-endosomal insulin degradation with the fact that IDE is primarily located in the cytosol [119,135–141] and therefore is not readily available for insulin located in endosomes.

An alternative explanation of the available data is that the endosomes may contain an insulinase that may share some of the characteristics of IDE. Pease et al. [107] found that endocytosed insulin was degraded *in vitro* by an enzyme having properties similar to IDE. For instance, it was inhibited by bacitracin but not by leupeptin and pepstatin. However, the rate of intravesicular insulin degradation was insensitive to a number of permeant thiol reagents that strongly inhibited IDE.

Authier et al. [141] have recently characterized an acidic thiol metalloproteinase in endosomes that seems to fulfill the requirements for such an insulinase. The protease was affinity-purified from endosomes and was shown to be different from IDE by several criteria: it had a lower pH optimum (4–4.5) and its activity was not influenced by inhibitors of IDE such as EDTA and NEM. IDE, on the other hand, could not be detected in the fractions by means of specific antibodies. Subcellular fractionation revealed that IDE was present in the S-fraction and in peroxisomes [141]. The newly described enzyme was not inhibited by leupeptin and pepstatin, in agreement with the finding that insulin degradation is unaffected by such inhibitors.

Studies with isolated hepatocytes and intact liver have shown that degradation of glucagon is similar to that of insulin [125]. Authier and Desbuquois [142] showed, using subcellular fractionation, that glucagon *in vivo* accumulated in endosomes. Degradation of labelled endocytosed glucagon could be demonstrated in endosomal fractions at low pH values, and at neutral pH in the presence of ATP. The pH optimum for glucagon degradation was even lower than that of insulin. At low pH, glucagon dissociates from its receptor and this dissociation seems to be required for its degradation. Authier and Desbuquois [142] found that various protease inhibitors affected insulin and glucagon degradation similarly. Degradation of either hormone was unaffected by phenylmethanesulphonyl fluoride and benzamide. It is therefore likely that both insulin and glucagon are degraded by the same endosomal thiol proteinase. This enzyme requires a metal ion and low pH for optimal activity. Glucagon may, in addition, be degraded at the plasma membrane as a glucagon-degrading protease has been detected in the plasma membrane fraction [110,143–146].

Analysis of endosomal fractions by means of Percoll-density-gradient centrifugation has suggested that two types of endosomes with different buoyant densities may be involved in degradation of both glucagon and insulin. The lower-density fractions containing insulin or glucagon are enriched in Golgi markers but they can be separated from the Golgi compartment by the DAB-density-shift method [6]. The low- and high-density components may represent early and late endosomes that are

sequentially involved in glucagon/insulin degradation [114, 132,142,147–151].

Endosomal degradation of EGF

Whereas insulin and glucagon seem to be completely degraded in prelysosomal compartments of the endocytic pathway, other peptide hormones are subject to partial proteolysis in early and late endosomes, before complete degradation in lysosomes. Rapid and limited proteolysis of EGF in endosomes have been documented in fibroblasts [94–97] and in rat hepatocytes [93]. In Rat-1 fibroblasts it was found by isoelectric focusing that the endosomal treatment of EGF leads to the formation of three distinct acidic forms in a sequential fashion [96,97]. The first degradation intermediate is formed by removal of the C-terminal arginine residue from the intact EGF. Subsequently four additional C-terminal amino acids are removed, leaving a C-terminal lysine residue which is subsequently removed. Precise information about the sites in the endocytic pathway at which limited and complete degradation of EGF takes place has been obtained using the isolated perfused rat liver as an experimental system [93].

Studies of the endocytic pathway in rat hepatocytes using electron microscopy have revealed that various endocytic tracers such as ¹²⁵I-EGF and ¹²⁵I-asialo-orosomucoid first accumulate in tubulovesicular structures located near the cell periphery [129,152,153]. These early endosomes contain ATP-driven proton pumps and are weakly acidic. Endocytosed ligands are subsequently transferred to late endosomes consisting of multivesicular structures located in the perinuclear region [16,154,155]. After 15–30 min the ligands, with or without receptors, enter lysosomes where complete degradation takes place.

By means of subcellular fractionation in e.g. sucrose or Nycodenz gradients it is possible to separate endosomes from lysosomes. Furthermore, since the kinetics of the intracellular transport of a given ligand such as EGF are known (within limits) it is possible to prepare endosomal fractions that are known to contain the ligand in early or late endosomes. Such fractions can be used to follow the proteolysis of the EGF in cell-free systems *in vitro*. The transport of ligand/receptor from early, peripheral endosomes to perinuclear, late endosomes is dependent on microtubuli and can therefore be blocked by microtubular poisons such as colchicine [45]. Another way of slowing the entry of ligand into late endosomes is to perform the experiments at reduced temperature [62,156]. Renfrew and Hubbard [93] showed that EGF is first processed in early endosomes by a carboxypeptidase B-like protease and is further processed in late endosomes by a trypsin-like protease and again by a carboxypeptidase B-like protease. The late endosomal step could be inhibited by reducing the perfusion temperature to 16 °C. These biochemical data were supported by electron microscopy of EGF–HRP cytochemistry. At low temperature the EGF–HRP was seen in peripheral tubular structures whereas multivesicular structures in the perinuclear area were labelled following prolonged perfusion. Complete degradation took place mainly in lysosomes.

The functional significance of the sequential processing of EGF in early and late endosomes, if any, is not known. EGF stimulates intrinsic tyrosine kinase activity of the EGF receptor upon binding and via a series of events stimulates DNA synthesis and cell division [112,157,158]. One possible role of the limited proteolysis may be to modulate the tyrosine kinase activity by modifying the ligand so that it is released from the receptor [98].

Endosomal activation of PTH

PTH is also subject to limited proteolysis in macrophage endo-

somes [90,100–102], but in this particular case the endocytic uptake leads to activation rather than termination of the signal. PTH is an 84-amino-acid single-chain polypeptide synthesized in the parathyroid gland. It has been suggested that the intact PTH must be metabolized to smaller fragments in order to activate target cells; intact PTH does not stimulate bone resorption [159–162]. Only fragments derived from the N-terminal regions of PTH are biologically active. Such fragments are formed mainly in the liver following secretion of the 84-amino-acid polypeptide from the parathyroid gland [163,164]. The hepatic metabolism leads to the formation of a number of peptides including an N-terminal fragment, PTH (1–34), which can stimulate adenylate cyclase in target cells [100]. It has been found that PTH is taken up selectively by hepatic macrophages, the Kupffer cells [102,163,164]. The fact that biologically active PTH fragments were released from the cells suggested that the Kupffer cells must be able to take up native PTH, partially degrade it and then release the biologically active N-terminal peptide fragment. *In vitro* studies have found that cathepsin D is likely to be the protease responsible for the liver endosomal proteolysis of PTH in Kupffer cells [100,163–165]. It has been shown that Kupffer cells express high cathepsin D activity [166,167]. Stahl and co-workers [90] demonstrated that rabbit alveolar macrophages were able to take up PTH, partially degrade it and release a bioactive fragment, PTH (1–34). The protease responsible was likely to be cathepsin D since degradation was blocked by pepstatin. Subcellular fractionation combined with *in vitro* studies of degradation of PTH in endosomal vesicles demonstrated that the metabolism of the hormone took place in endosomes and not in lysosomes or at the plasma membrane [90]. The mechanism whereby the PTH fragment is retro-endocytosed from endosomes is not known. PTH uptake is via a high-capacity, low-affinity mechanism which is different from the high-affinity binding in target cells. Diment et al. [90] reported that the uptake in macrophages is not inhibited by the PTH fragment [168,169]; it is therefore unlikely that the active hormone is transported back to the plasma membrane in association with the receptor, unless its affinity increases at low pH. Macrophages show a very extensive retroendocytosis of fluid-phase markers, indicating that a large proportion of the fluid in the early endosomes is released back to the extracellular medium [170]. PTH fragments may therefore to a large extent be released by a non-specific retroendocytic mechanism.

ROLE OF ENDOSOMAL PROTEOLYSIS IN ANTIGEN PRESENTATION

Antigens are categorized as exogenous or endogenous depending on whether they are derived from outside or synthesized within the cell. Two different classes of polymorphic major histocompatibility complex (MHC) molecules have evolved to deal with endogenous peptides formed in the cytosol and exogenous peptides formed in the endocytic pathway, MHC class I and class II. As endogenous antigens bind MHC in the biosynthetic pathway [i.e. the ER] we will only consider MHC class II in this review. MHC class II consists of an α and a β chain, which are transmembrane molecules that in ER rapidly associate with a non-polymorphic invariant chain (Ii) [171,172]. Invariant chain is believed to form a trimer which again associates with three class II molecules forming a nanomeric complex (for a review see [173]). The proteins within the complex undergo an extensive 'quality control' in the ER and various post-translational modifications which may be the cause of the observed delayed transport from ER to the plasma membrane and endosomes [173,174]. The invariant chain contains two leucine-based signals

in its cytoplasmic tail that direct the MHC class II to endosomes [175–179] either directly from the *trans*-Golgi [174,180] or via the plasma membrane [181] (Figure 2). The presence of invariant chain is, in many cases, essential for the ability of a cell to present processed antigen via MHC class II [182–186].

An important function of the invariant chain is to prevent premature binding of peptide to MHC class II both in ER and later in the biosynthetic and endocytic pathway [176,187,188]. If class II molecules were to leave ER with unoccupied binding groove, this could lead to interactions with segments of endogenous protein. Avoidance of such binding is mediated by the invariant chain both in the ER and later in the biosynthetic and endosomal pathways [189–191]. In order to explain antigen processing and presentation by MHC class II molecules the following points have to be dealt with: (a) how is the invariant chain removed from the peptide-binding groove such that the antigenic peptide may bind? (b) Where in the endocytic pathway do the MHC class II molecules meet exogenous antigens and where does the binding of peptide take place? (c) By which proteolytic mechanisms are the antigens partly degraded such that defined peptides are formed at the same time as complete degradation is prevented and what kind of proteases are required? (d) How is the antigen taken up in antigen-presenting cells and is there a specific sequence of proteolytic steps?

Roche and Cresswell [189] showed that dissociation of the invariant chain releases class II molecules competent to bind peptides and that *in vivo* proteolysis by cathepsin B, a protease present in endosomes, releases peptide-receptive class II molecules from their association with the invariant chain. This observation suggested that invariant chain removal *in vivo* may depend on proteolysis. The mechanism for invariant chain removal may differ kinetically in different cells. However, it is likely that proteolysis results first in the sequential formation of processing intermediates, including the transmembrane region of Ii [192–194]. At the later stages CLIP, a part of the invariant chain, seems to block the access of immunogenic peptides by occupying the peptide-binding groove [190,191,195].

Analysis of the intracellular distribution of invariant chain as well as MHC class II by immunofluorescence and electron microscopy of cryosections shows that invariant chain and MHC class II are located in early endosomes, endocytic carrier vesicles and prelysosomes [177,193,196]. Recent studies indicate that both MHC class II and invariant chain are already introduced into the endocytic pathway at the level of the early endosomes [181,196a]. During the intracellular transport the invariant chain loses increasing portions of the C-terminal side but is still associated with class II molecules [192–194]. The thiol protease inhibitor, leupeptin, was found to stop degradation of Ii at p22, indicating that further processing is dependent on thiol protease activity. In the studies above neither MHC class II nor invariant chain were detected in lysosomes and the processing of invariant chain is therefore likely to take place in prelysosomal compartments. Zachgo et al. [197] analysed the morphological effects of leupeptin in detail in melanoma cells. It was found that p33 Ii was present in early endosomes, endocytic carrier vesicles and prelysosomal vesicles. Therefore, the leupeptin-insensitive proteolytic processing of p33 Ii probably takes place in endocytic carrier vesicles or at least begins in the endocytic carrier vesicles and continues in the prelysosomes. Processing of p22 Ii is necessary for achieving binding of peptides to MHC class II and the subsequent expression of MHC II on the cell surface [198]. However, Zachgo et al. [197] showed that leupeptin inhibited general transport of endocytosed material along the endocytic pathway. This effect was exerted on the transport from multivesicular carrier vesicles to the prelysosomal compartment,

Figure 2 Intracellular transport of MHC class II–II molecules

Exogenous antigens are internalized by endocytosis and degraded to peptide antigens, primarily in late endosomes. Class II α and β chains are associated with trimeric invariant chain in the ER, forming a nonameric complex. Here, Ii both prevents premature peptide binding and stimulates ER exit of class II. Invariant chain–class II nonamers are transported through the Golgi complex and may enter the endocytic pathway by two different routes. The secretory route implies rapid internalization of the invariant chain–class II complex. The complexes may also enter the endocytic pathway by a direct route. Following delivery to endosomes, invariant chain is cleaved and removed from class II molecules, a process involving several proteases. HLA-DM is essential for the removal of CLIP, a luminal region of Ii which is bound to MHC class II late in the endocytic pathway. Removal of Ii and its degradation products (including CLIP) allows association of antigenic peptides to class II dimers. The formation of peptide–class II complexes is suggested to take place in a late endocytic compartment; MHC class II Compartment (MIIC) or Compartment for Peptide Loading (CPL), and is accompanied by a conformational change in the class II molecules, resulting in resistance to SDS at room temperature. Peptide–class II complexes are finally transported, presumably from CPL, to the plasma membrane for presentation to CD4⁺ T-cells.

resulting in an increase in the number of endocytic carrier vesicles. A similar effect of leupeptin has been demonstrated previously in rat hepatocytes [199,200]. Together with the direct effect of leupeptin on antigen processing, this shows that this drug may have complex effects.

Newer data indicate that invariant chain itself may regulate maturation of endocytic vesicles [201,202]. It was shown that invariant chain induces formation of large endosomes in transiently transformed COS and CV1 cells expressing high levels of the protein. These organelles were transferrin-accessible and were therefore considered early endosomes. Furthermore, anterograde movement of both a fluid-phase marker (ovalbumin) and transfected lamp-1 was retarded and the effect of invariant

chain on the kinetics of endocytic transport required signals in its cytoplasmic tail [201]. These alterations in endosomal structure and function were seen after high-rate expression of invariant chain obtained by transient transfection. However, isolated Langerhans cells expressing invariant chain are also found to contain large lucent acidic vacuoles with the characteristics of early endosomes [203–205]. Permanent cell lines transfected with Ii also contain similar large Ii-induced structures with properties of early and late endosomes as well as lysosomes (E. Stang and O. Bakke, unpublished work). Furthermore it is also interesting to note that in Ii-transfected cells not showing the large vesicular structures the transport of endocytosed HRP from early to late endosomes is dramatically delayed [196a]. These studies dem-

onstrate that endogenous molecules may modulate endocytic transport like inhibitors of proteolytic enzymes [197,200]. Katunuma et al. recently pointed out that invariant chain shares a high level of sequence similarity with the cystatin family, which are endogenous cysteine protease inhibitors [206]. They also found that isolated Ii was indeed actively inhibiting cysteine proteases. Such a property might, in principle, lead to the observed alterations of the endocytic pathway.

The design of the peptide-binding grooves are different in the MHC class I and class II molecules [207]. In MHC class I the groove is closed at both ends, and the free NH_2 and COOH groups of the peptide bind to residues at the very ends of the groove [208–210]. These features of interactions between class I molecules and peptides limit the length of peptides to 8–10 residues. Class II molecules, on the other hand, do not make bonds with the terminal groups of the peptide. The ends of the groove are open and the class II-associated peptides can extend outside the groove [195,211–214]. As a result, class II-associated peptides may have a much broader range of lengths.

Protein antigens may be internalized in antigen-presenting cells by fluid-phase endocytosis or by receptor-mediated endocytosis. Receptors involved include immunoglobulins on B-cells that recognize epitopes on antigens [215,216]. After internalization the antigens may be processed in the endocytic pathway and peptides derived from such antigens are subsequently presented at the surface of B-cells. The B-cell surface immunoglobulins will thus serve to capture and concentrate antigens within the cells. Receptor-mediated uptake is much more efficient than fluid-phase uptake. A B-cell that expresses a specific antibody for a given antigen is 100–10000-fold more efficient in acquiring antigen compared with B-cells with irrelevant surface antibody [188]. The unfolding and denaturation that is needed for antigen presentation is induced by low pH in endocytic compartments. In accordance with this, agents that raise pH in these compartments, such as ammonium chloride and chloroquine, inhibit presentation of peptides to B-cells by class II molecules [217]. Low pH is also necessary for the acid hydrolases that are involved in antigen processing.

Whether class II molecules and invariant chain reach endosomes directly from TGN or via the plasma membrane is still a matter of debate (for a review see Sandoval and Bakke [218]) (Figure 2). It seems, however, established that invariant chain and class II molecules co-localize early in the endocytic pathway [177,193,196] (E. Stang and O. Bakke, unpublished work) making it likely that proteolysis of antigens takes place at the same time as the invariant chain is degraded and released from MHC class II. The proteolytic activity in the endocytic compartment is necessary for denaturation and antigen processing. On the other hand, complete degradation of antigens must be prevented. These problems are illustrated by the paradoxical effects of leupeptin which can enhance protection of peptides from ovalbumin bound by class II molecules [219]. Such an effect of a protease inhibitor indicates that endosomal proteases have the capacity to both generate and destroy immunogenic peptides [220]. A possible mechanism whereby antigenic peptides are protected is by antigen binding to MHC class II. Peptides may be shielded from total degradation by rapid binding to the peptide-binding groove of MHC class II. Mouritsen et al. [221] exposed antigenic peptides to various proteolytic enzymes (cathepsin B, pronase E) in a cell-free system either with or without peptide-binding MHC class II molecules present. They found that an antigen fragment, once bound to class II molecules, was effectively protected against proteolytic destruction. Binding of peptide to MHC class II also results in an increased stability of the class II $\alpha\beta$ heterodimer in the presence of SDS and can be

visualized by SDS/PAGE [222]. The class II molecules may protect only the core epitope of the peptide, both peptide termini appear to be exposed and may therefore be trimmed. It was shown, for instance, that class II-bound peptide, which was protected against cathepsin B, could still be modified by the exopeptidase, aminopeptidase N [221].

Information about the proteases involved in antigen processing has been obtained in three ways: (a) antigen-presenting cells have been incubated with various protease inhibitors to determine whether a given protease or group of proteases is involved in the steps leading to antigen presentation; (b) processing of antigen has been carried out *in vitro* in the presence of the relevant enzymes and/or inhibitors; (c) the intracellular locations of the interacting components (MHC II, invariant chain, proteases) have been determined by means of immunocytochemistry. Incubation of cells with protease inhibitors results in a multitude of cellular effects and the results obtained are prone to misleading conclusions. For instance, cathepsin B is involved in self-activation as well as processing of invariant chain. Inhibition of antigen presentation by leupeptin may therefore be an indirect effect independent of any effect of cathepsin B on antigen processing/proteolysis. In addition, leupeptin may inhibit specific steps in the intracellular trafficking as discussed above.

Although it has been established that processing of exogenous antigens involves proteolysis, the specific proteases involved have been identified for only a few antigens. It seems likely that distinct immunogenic peptides from a single protein may be generated by the action of different sets of proteases. Several studies have shown that thiol proteases play a central role in processing. Takahashi et al. [223] studied proteolytic formation of three T-cell epitopes (defined by three T-cell clones) and used a panel of inhibitors to determine the role of each of four classes of proteases. For all epitopes it seemed that thiol proteases (cathepsin B, cathepsin L) were the only proteases required. In support of this, it was found that incubation of myoglobin with cathepsin B leads to the formation of peptides that can be presented to T-cells without further processing. A similar crucial function of thiol proteases has been suggested by other reports [198]. It is, however, likely that cathepsin D also plays an important role in antigen processing. Van Noort et al. [224] found that fragments formed from sperm whale myoglobin during incubation with partially purified macrophage endosomes were identical to those generated after uptake *in vitro*. Two enzymes, cathepsin B and cathepsin D, could account for all cleavages. It was found that cathepsin D was responsible for the initial cleavage and the fragments released contained most epitopes for murine myoglobin-specific T helper cells. The T-cell epitopes were located at the N-termini of the fragments. Conceivably, a structural relationship between antigen processing by cathepsin D and antigen recognition by class II molecules may exist, i.e. part of the cathepsin D-recognized motif (the N-terminal part) may have structural features that are recognized by different class II molecules. The important role of cathepsin D is compatible with results indicating that inhibition of cathepsin D (by pepstatin A) in macrophages blocks endosomal degradation of BSA [86] and PTH [90]. Cathepsin D has also been shown to release T-cell epitopes from ovalbumin [225]. Recent data also indicate that the initial cleavage of the invariant chain (in B-lymphoblastoid cell lines) is catalysed by an aspartic protease whereas a cysteine protease catalyses the final stages of invariant chain release [226].

The relative roles of various endocytic compartments in peptide binding to class II molecules have not been clearly established. The site where class II molecules bind peptide and where partial proteolysis of antigens takes place may differ for different

antigens. Both endosomes and lysosomes function as processing compartments as peptide fragments have been found in both organelles. Antigen processing may commence in early endosomes, but some antigens may require more harsh conditions. Disruption of disulphide bonds takes place in late lysosomal compartments [227]. An interesting approach to studying the possible role of early endosomes in antigen processing has been to allow uptake of antigen (e.g. cytochrome *c* or ovalbumin) via the transferrin receptor. An antigen was coupled to transferrin and was internalized into antigen-presenting cells (B-lymphoma cells) via the transferrin receptor. The uptake rate of the antigen was much higher than the uptake of antigen in the fluid phase. It was even possible that receptor-mediated uptake and fluid-phase uptake were via different endocytic pathways [228,229]. It was shown that the antigen moiety of the complex was degraded mainly in early endosomes. A T-cell response to the conjugate was elicited that was consistent with an early processing compartment.

Although easily degradable antigens such as ovalbumin and lipopolysaccharide may be processed and even bound to MHC class II in early endosomes [228,229], several observations point to lysosomes as a main site of antigen processing. Reduction of antigen sulphide bonds is required for presentation of some epitopes [227] and appears to take place in lysosomes. Harding and co-workers [230–233] prepared antigens encapsulated in liposomes that released their contents either in endosomes or in lysosomes and found that the lysosomal processing was more efficient than that taking place in endosomes [234]. Antigen processing is inhibited at 18 °C, also suggesting that a late compartment is required for effective antigen presentation [62]. The notion that lysosomes are a main site of production of immunogenic peptides is, on the other hand, difficult to reconcile with other observations that lysosomes lack class II molecules [193] (E. Stang and O. Bakke, unpublished work). An explanation of these apparent inconsistencies is that the class II molecule-deficient organelles are a subgroup of lysosomes (see below) or that the processing compartments vary with cell type. In conclusion, the endocytic pathway in antigen-presenting cells may be considered a digestive tract rather than a transport pathway to terminal lysosomes. The conditions for proteolysis vary from primary endosomes to late lysosomes with respect to pH and enzyme composition. Various antigens, depending on their structure, may be processed in early and late parts of the endocytic pathway. Since MHC class II molecules are present all along this digestive tract (with the possible exception of lysosomes), antigenic peptides may be bound at the site of their formation.

Following peptide loading of class II molecules the peptide–class II complex is transported to the cell surface for presentation to T-cells (Figure 2). The pathway followed by the complex is not known. However, reports have characterized endosome-related subcellular compartments that probably are part of this exocytic pathway [180,235–238]. Such a compartment may also be a site for peptide loading and was therefore termed CPL (compartment for peptide loading) [239] (Figure 2). The CPL has been identified by subcellular fractionation and by immunocytochemistry. These studies have taken advantage of the fact that peptide loading of the class II complex renders the complexes SDS-stable [240]. It is therefore possible to determine the distribution in subcellular fractions of peptide-loaded complexes. The studies showed that a class II-containing intracellular compartment could be resolved from both early endosomes and dense lysosomes. The invariant chain was absent or rapidly degraded in the CPL and functional SDS-stable class II–peptide complexes could be detected. These were believed to appear subsequently on the cell surface and

stimulate T-cells. Endocytic markers entered CPLs only to a limited extent; the bulk of endocytosed material was transported to conventional late endosomes/lysosomes. The CPLs in B-cells contain internal membrane and look like MVBs. It is reasonable to assume that the class II/Ii are delivered to early endosomes together with antigens and lysosomal hydrolases. The CPL may then bud off from early endosomes, similar to the endocytic carrier vesicle described earlier [18].

A main function of the newly discovered CPL may be to coordinate proteolysis of Ii and antigens such that unoccupied receptive class II complexes are available at the same time as antigenic peptides are produced. It is reasonable to assume that Ii is degraded during a shorter time span than the various antigens. As commented by Schmid and Jackson [239], the CPL may provide an environment in which peptide- and Ii-free class II molecules are held in a receptive state (the ‘floppy’ conformation) at the same time as antigens are degraded and loading occurs. Furthermore, *in vitro* studies have shown that unoccupied class II molecules aggregate and that the aggregates dissolve upon addition of appropriate peptides [188]. Such a mechanism in the CPL could ensure that only loaded class II molecules are released to the cell surface.

Molecules that are essential for loading antigen in the MHC class II peptide-binding groove and possibly the proper transport to the plasma membrane, are the α and β chains of HLA-DM [241], the MHC-encoded molecules that can restore peptide presentation defects in mutant cell lines [242,243]. In cell lines missing the HLA-DM molecules the CLIP peptides are not removed from class II molecules and peptide binding is obstructed. Recently E. Mellins and co-workers have obtained antibodies recognizing the CLIP–class II molecule and morphological studies show that this complex accumulates late in the endocytic pathway before the molecules appear at the plasma membrane (E. Stang, O. Bakke and E. Mellins, unpublished work). These observations thus indicate that MHC class II may be routed to the plasma membrane via a compartment late in the endocytic pathway.

EARLY AND LATE LYSOSOMES

Lysosomes have, since their discovery, been regarded as heterogeneous organelles. However, degradation of endocytosed and autophagocytosed material has been assumed to take place in one functional homogeneous group of organelles, the lysosomes. It has become clear that degradation may indeed take place prelysosomally, in endosomes. Recent data indicate that the lysosomal degradation may take place sequentially in two groups of lysosomes. It has been known for several years that the late endocytic compartments consist of at least two types of digestive organelles, called late endosomes and lysosomes. The late endosomes express high concentrations of M6PR [197] and may contain a spectrum of different proteases. The functional distinction between late endosomes and lysosomes may therefore not be very sharp and the two groups of organelles should rather be termed early and late lysosomes to emphasize their catabolic properties. The function and structure of the early lysosomes may differ from cell type to cell type and may also depend on the endocytic and autophagic activity of the cells.

Degradation of endocytosed molecules in rat hepatocytes may take place prelysosomally, in endosomes, as discussed above. In addition, the lysosomal degradation of endocytosed ligands may be initiated in an active subgroup of lysosomes which may also be termed ‘early lysosomes’. Degradation of autophagocytosed material may be initiated in the same subgroup of early lysosomes which can be separated from other lysosomes in Nycodenz

Figure 3 Three models to explain exchange between early (EL) and late lysosomes (LL)

Endocytosed substrate is assumed to be introduced into the lysosomal system via an endocytic carrier vesicle (ECV) (but could in principle be replaced with a multivesicular endosome or an autophagosome). In model I the ECV fuses with a late lysosome whereby an early lysosome is formed. A lysosome buds off from the early lysosome, and after a maturation process this lysosome is ready for fusion with another ECV. In model II the ECV fuses with a late lysosome which matures into a late lysosome. The late lysosome is then ready to fuse with another ECV. The third model (model III) is a 'vesicle shuttle' model. The early and late lysosomes are assumed to be stable organelles that exchange material by vesicle shuttling back and forth between the two populations of lysosomes. The early lysosomes receive substrate from the ECV.

gradients because of their low buoyant density [244,245]. The early lysosomes in hepatocytes are a meeting place for endocytosed and autophagocytosed material and may therefore be called 'amphisomes' [246]. Wattiaux and co-workers were able to demonstrate that degradation of both ^{125}I -TC-labelled formaldehyde-treated albumin (in liver endothelial cells) and ^{125}I -TC-labelled asialo-orosomucoid (in liver parenchymal cells) mainly took place in organelles that had not been affected by density perturbants such as Triton WR-1339 or invertase [49,60]. The degradation products trapped in the early degradative compartments were relatively slowly transferred to the lysosomes that contained the density perturbants. It was suggested that lysosomal degradation took place in two serially connected compartments: transfer lysosomes (early lysosomes) and accumulation lysosomes.

Similar data were obtained in *in vivo* and *in vitro* studies of endocytosis via the mannose receptor in liver endothelial cells. It was found that degradation of mannosylated albumin was initiated in a buoyant, electronlucent group of early lysosomes and that the degradation products slowly appeared in denser lysosomes. These denser lysosomes could be made even denser by allowing endocytosis of the density perturbant yeast invertase [57]. By giving the cells a pulse of ligand attached to 5-nm-diam. gold particles and then after overnight chase adding ligand labelled with 10-nm-diam. gold particles it was found that it took

several hours before the two labels co-localized in the same lysosomes.

Two functional groups of lysosomes (connected in series) may also be present in non-hepatic cells. For instance, the relation between so-called tubular lysosomes [247] and the dense lysosomes in macrophages is reminiscent of the dual lysosomal systems in liver endothelial cells and parenchymal cells. There seem to be tubular lysosomes and vacuolar (dense) lysosomes involved in early and late degradation respectively [18,248]. The dense lysosomes, which may be formed from the prelysosomes by a budding process or by a maturation process [18], do not seem to represent a dead end of the endocytic pathway. Endocytosed, undegraded material from long-lived endocytosed markers are invariably distributed in both prelysosomes and lysosomes [249,250]. These data and the finding that subcellular distribution of the membrane protein Igp 120 in the membranes of the prelysosomal compartment and in the lysosomes is quantitatively similar rather argues for some kind of dynamic equilibrium between the two compartments.

Recent studies using ^{125}I -TC-labelled ligands to identify degradative compartments in J774 macrophages have supported the notion that early and late (communicating) lysosomes are involved in degradation of endocytosed ligands (T. Tjelle, unpublished work): ^{125}I -TC-labelled ovalbumin (endocytosed via the mannose receptors) in J774 macrophages is degraded in a prelysosomal (or early lysosomal) compartment. The degradation products are subsequently transferred to lysosomes that may be rendered denser by perturbants such as colloidal gold. Interestingly, it does not seem possible to get a complete transfer of labelled TC from the prelysosomes to the dense lysosomes. Studies using ligand labelled with colloidal gold have led to the same conclusion: although gold is found sequentially in prelysosomes and lysosomes it is not possible, even after long chase periods, to empty the prelysosomes [250,251]. These observations suggest that material may be recycled back from the dense lysosomes to the prelysosomes.

Harding and Geuze have recently described a group of 'early lysosomes' in B-cells [235] and in macrophages [83,231] that contain MHC class II. Antigen processing, as well as binding of peptides, takes place in these early lysosomes that have an earlier kinetic position on the endocytic pathway than the terminal vacuolar, dense lysosomes. Endocytic markers such as colloidal-gold-labelled BSA are found in tubular lysosomes before they enter terminal lysosomes. The MHC class II-containing early lysosomes described by Geuze and Harding [83,252] are likely to be related to late endocytic compartments described earlier in macrophages: tubular lysosomes [247,253,254] and late endosomal tubulovesicular compartments [18]. The terminal lysosomes may degrade antigens that have escaped destruction in the early lysosomes, and peptides may conceivably be recycled from the terminal to the early lysosomes where binding to MHC class II may take place. A recycling pathway from terminal lysosomes to early lysosomes/prelysosomes has been suggested by Griffiths and co-workers [251] and by Berg and co-workers [255].

Three related models may be envisaged to explain this recycling (Figure 3): first, the dense lysosomes may bud off from the prelysosomes, and, following some sort of maturation during which degradation of slowly degradable substances is completed, the lysosomes will again be ready to fuse with the prelysosomes; secondly, the prelysosomes may arise by fusion between dense lysosomes and endosomes, and the prelysosomes then gradually mature into dense lysosomes that are ready to fuse with new endosomes; thirdly, the prelysosomes and the lysosomes are relatively stable compartments that communicate by vesicle shuttling. The prelysosomes receive input from endocytic carrier

vesicles and (during nutritional step-down conditions) from autophagosomes.

CONCLUSIONS

Degradation of endocytosed or autophagocytosed macromolecules has been considered to be a lysosomal event. It is now clear that lysosomal hydrolases are introduced into the endocytic pathway at an early stage and it is therefore possible that partial or even complete hydrolysis of macromolecules may take place in prelysosomal compartments. The endocytic pathway should therefore be considered as a digestive tract rather than a transport pathway.

Available data suggest that an endocytosed macromolecule may experience limited or complete degradation in several different ways: (1) the ligand may be partly degraded at the plasma membrane; (2) the ligand may be completely degraded in the endosomes; (3) the ligand may experience limited proteolysis in early and late endosomes and subsequently the remainder of the ligand may be completely degraded in lysosomes; (4) the ligand may be partly degraded in endosomes and then be released by retroendocytosis; (5) the ligand may be transported unmodified through the endosomal pathway to the terminal lysosome where complete degradation takes place; and (6) the ligand may be modified (i.e. by low pH, and/or by limited degradation) in the endocytic compartment in such a way that it is translocated through the endosomal membrane to reach the cytosolic compartment [256].

The diverse proteolytic processing of ligands in the endocytic pathway may depend on a series of factors as detailed below. (1) Only a limited set of proteases may be active in the early endosomes. (2) The pH in endosomes is optimal for only some enzymes. (3) Local conditions in endosomes may influence the activity of certain enzymes. For instance membrane-bound cathepsin D in early endosomes may express a higher pH optimum than the soluble enzyme in lysosomes [257]. Cathepsin B may act both as an endopeptidase and an exopeptidase. The pH optimum is lower for the exopeptidase than for the endopeptidase [258,259]. (4) Different endocytic pathways may transfer ligands to different proteolytic environments. At least in some cells endocytosis may take place both via coated pits (clathrin-dependent endocytosis) and outside coated pits (via clathrin-independent endocytosis) [1,4,5,260–266]. The endosomes arising from the two origins may differ with regard to content of enzymes and proton pumps. Paul H. Weigel and co-workers have shown that the galactose receptor in rat hepatocytes exist in two different states [267–271]. The two types of receptors (termed 'state I' and 'state II') operate in parallel and mediate uptake of ligand into two different types of endocytic pathways. The state I receptor mediates uptake into endocytic compartments in which degradation is initiated without a delay. State II receptors, in comparison, mediate uptake into a classical endocytic pathway where degradation is initiated after a lag phase that probably corresponds to the transit time of the ligand through early and late endosomes.

The distribution of proteases in the early and late endosomes, as well as a pH gradient, makes possible a restricted and controlled proteolysis. This would not be possible in the harsh environment of a terminal lysosome. The restricted endosomal degradation is utilized in particular for activation and inactivation of proteins and for processing of antigens.

A major function of endosomal proteolysis is activation (lysosomal proteases, PTH) and inactivation (insulin, glucagon, growth factors) of proteins. A key event in this context is activation of lysosomal proteases such as cathepsin D and

cathepsin B [272]. Cathepsin D seems to play a particularly important role in endosomal proteolysis. The endosomal form of cathepsin D may be a membrane-associated form of higher molecular mass than the cathepsin D found in lysosomes [20]. It is autocatalytically activated and activates other lysosomal enzymes as well. Cathepsin D plays a crucial role in the activation of PTH in Kupffer cells [100,165] and may initiate the proteolytic processing of invariant chain in endosomes [89]. A particularly striking example of a regulatory role of cathepsin D in endosomal proteolysis is seen in oocytes. Studies in *Xenopus* oocytes have indicated that vitellogenin (VTG) undergoes cleavage into large fragments within an endocytic compartment termed 'light yolk platelets'. These organelles are precursors to 'heavy yolk platelets' which are the final storage compartment. The proteolytic processing is inhibited by pepstatin A, suggesting that cathepsin D is involved in the VTG cleavage [273–275]. Yolk formation in chicken oocytes is similar to that in *Xenopus*. In chicken the bulk of yolk is derived from both plasma VLDL and from VTG which are partially cleaved prior to storage. Again, the proteolysis is inhibited by pepstatin A. Schneider and co-workers demonstrated that cathepsin D purified from oocytic yolk generated fragments from VLDL and VTG that were indistinguishable from those found in yolk platelets [276]. Cathepsin D is therefore a key enzyme for yolk formation.

The restricted endosomal proteolysis is instrumental in connection with antigen presentation. The proteolytic activity in the endosomal compartment is necessary for denaturation and processing of antigens whereas complete degradation must be prevented. The endosomal pathway at the same time co-ordinates the proteolysis of antigens and the invariant chain bound to the class II complex.

Recent studies have suggested that even the lysosomal phase of intracellular degradation should be subdivided into an early and a late step. This phenomenon has been observed in macrophages [18], liver endothelial cells (R. Kjekshus et al., unpublished work) [277] and in rat hepatocytes [245]. The main degradation takes place in the early lysosomes, whereas undegradable and less degradable materials are transferred to late lysosomes. It has been suggested that material may be recycled back from late to early lysosomes [18,278]. The functional significance of a two-step degradation could be that two groups of organelles acting in series may be an effective digestive system that readily adapts to changes in the load of substrate brought in by endocytosis. By transferring degradation products or undegraded substrate to the denser lysosome, the first degradative organelle is always available for new substrate. A recycling between late and early lysosomes could be useful for antigen presentation. Some of the antigens may need the harsh environments of the terminal lysosomes to be processed. The peptides formed may be recycled to the early lysosomes where the class II MHC molecules are present [231].

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