Minireview

Endosomal proteolysis of internalized proteins

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Abstract Endosomal proteases have been implicated in the degradation of internalized regulatory peptides involved in the control of metabolic pathways and in the processing of intracellular antigens for cytolytic immune responses. Processing in the endocytic vesicles is regulated by changes in endosomal acidity due to the presence of an ATP-dependent proton pump which modulates protease activity, protein unfolding and receptor-ligand interactions. A limited number of proteases appear to reside in endosomes which do not contain the full complement of active proteases capable of completely degrading all internalized polypeptides. Retention of some acid hydrolases in endosomes is apparently related to their association with undefined endosomal membrane receptors. The limited number of proteases and the pH gradient from neutral to acidic (pH 7 to 5) within endosomes make possible a selective and controlled processing environment in comparison to lysosomes. The full set of endo- and exopeptidases that break down proteins to amino acids are active later in the pathway in lysosomes.

Key words: Endosomes; Endocytosis; Major histocompatibility complex class II; Antigen processing; Endopeptidase; Cathepsin

1. Introduction

Two predominant cellular systems participate in the degradation of cellular proteins: the cytoplasmic ubiquitin pathway and the vacuolar pathway (including mainly lysosomes and endosomes). The lysosomes, with their broad-specificity proteases, were considered to be organelles where complete degradation occurs. Recently, the study of endocytosis has broadened the view that early and late endosomes are not only sites of sorting and segregation, but also represent a biologically important processing compartment within cells. Recent evidence also indicates that lysosomal hydrolases are introduced into the endocytic pathway at an early stage so that partial or even complete hydrolysis of macromolecules may take place in prelysosomal compartments.

Hormones, growth factors, toxins, hydrolase precursors and foreign antigens are cleaved by endosomal proteases. The restricted endosomal proteolysis is utilized both for inactivation (insulin, glucagon, epidermal growth factor (EGF), invariant chain (Ii), antibodies and vitellogenin) and activation (lysosomal hydrolases, parathyroid hormone (PTH), protein antigens and toxins) of internalized proteins. The intraendosomal cleavage of proteins following endocytosis has been studied in a large number of cells, especially hepatocytes, macrophages, B-lymphocytes, fibroblasts and oocytes. These studies have raised interesting questions regarding the extent of endosomal proteolysis in different cell types, the nature and specificity of endosomal proteases, the points in the endocytic pathway where acid hydrolases are delivered and the mechanisms by which proteases are permanently retained within endosomes. Furthermore, recent studies have demonstrated that the cysteine protease cathepsins B and H, and the aspartic protease cathepsin D play a particularly important role in endosomal proteolysis. The present review focuses on the endosomal pathways involved in the processing of polypeptide hormones and internalized antigenic proteins.

2. Proteolytic modifications of internalized proteins in endosomes

2.1. Early observations

Cell-free studies with intact endosomes by Diment and Stahl [1] and Pease et al. [2] provided indication that endosomes might be proteolytically active against internalized mannosylated bovine serum albumin (mannose-BSA) and insulin (Fig. 1). Subsequent investigations have implicated endosomal proteases in processing polypeptide hormones such as glucagon [3-6] and PTH [7], growth factors such as EGF [8], plant and bacterial toxins such as ricin toxin [9,10] and cholera toxin [11]. The conclusion that degradation of internalized ligands occurs or is, at least in part, initiated in endocytic vesicles is based on: (i) the high recovery of internalized ligands in purified endosomes by subcellular fractionation studies, while little association was observed with lysosomes [12]; (ii) the major accumulation of internalized hormones [3,12] and toxins [11] induced by acidotropic agents (i.e. chloroquine), while effecting a minor accumulation of these ligands in lysosomes; (iii) the extraction from endosomes of polypeptide hormone fragments of insulin [13], glucagon [3,4,6] and EGF [8] using a reverse-phase high performance liquid chromatography procedure corresponding to the in vivo sites of hormone hydrolysis; (iv) the degradation of endosomal ligands in cell-free endosomes incubated at low pH [1,2,4,8,14]; (v) the identification using morphological [15], biochemical [16] and immunological criteria [1,6,17,18] of soluble and membrane-associated endosomal hydrolases; and (vi) the demonstration using iodinated protein ligands and

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Abbreviations: EAI, endosomal acidic insulinase; ER, endoplasmic reticulum; Ii, invariant chain; MHC, major histocompatibility complex; EGF, epidermal growth factor; PTH, parathyroid hormone; HPLC, high performance liquid chromatography; TCA, trichloroacetic acid

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small synthetic fluorogenic substrates that endosomal hydrolysis is not equivalent to that found in lysosomes [19].

2.2. Properties of endosomal proteases

Using isolated intact endosomes containing internalized hormones, growth factors and toxins facilitated the characterization of the mechanisms of endosomal proteolysis [20]. The proteolytic activities directed against mannose-BSA, ricin A-chain, PTH and glucagon were maximal at pH 4–4.5 [1,4,7,9]. However, those directed against insulin and EGF displayed broad pH optima (pH 5–5.5), and were catalytically active at pH 7 [8,14]. Although mainly identified at low pH, endosomal proteolysis was detected at pH 7 if ATP, the substrate of the H^+-ATPase, was added to the medium [1,4,9,14]. The ability of ATP to stimulate endosomal proteases did not occur if the isotonic medium was depleted of Cl^-, as expected from the electrogenic properties of the endosomal proton pump [21]. The effect of ATP was also suppressed by weak bases (i.e. chloroquine and dansyl cadaverine), by proton ionophores (i.e. monensin, nigericin and valinomycin) and by inhibitors of the vacuolar-type H^+-ATPase (i.e. *N*-ethylmaleimide and dicyclohexylcarbodiimide) [1,4,9,14]. Thus, endosomal proteolysis is functionally linked to ATP-dependent proton pump-mediated acidification of the endosome lumen. Inhibiting the dissociation of insulin and glucagon from their respective endosomal receptors reduced ligand degradation [4,14,22]. This indicated that ATP likely stimulated endosomal proteolysis by promoting endosomal acidification with subsequent ligand-receptor complex dissociation and degradation of free luminal ligands by acid-optimal proteases.

2.3. Patterns of endosomal proteolysis

Because of the potential importance of endosomal proteolytic activities in terminating or otherwise in altering the hormone signal by the generation of biologically active degradation products, investigations have been carried out to determine the extent of endosomal hydrolysis. For some ligands, complete hydrolysis was observed, while for others, proteolysis appears to be limited (see Table 1). Large protein ligands, such as galactosylated-BSA or asialoorosomucoid, which require extensive endo- and exopeptidase actions to be converted into trichloroacetic acid (TCA)-soluble material are not significantly degraded [19]. A single cleavage of PTH-(1–84) at the peptide bond F^{34}–V^{35} to the bioactive fragment PTH-(1–34) has been identified in endosomes of rabbit alveolar macrophages, with a subsequent release of this physiologically relevant form in the cellular medium [7]. Degradation of tyramine-cellobiose-labeled asialofetuin in isolated rat hepatocytes begins in endosomes and is completed in lysosomes [23]. Other studies have documented that EGF undergoes limited proteolysis at its C-terminus within endosomes of human, rat fibroblasts and rat hepatocytes [8]. Three forms of EGF processed at their C-termini appear to be generated in a sequential fashion in endosomes: EGF-(1–52) which results from the removal of an arginine residue, EGF-(1–48) which results from the removal of a tetrapeptide W^{49}–W^{50}–E^{51}–L^{52} and in which a lysine residue exposed at the new C-terminus is removed to generate the EGF-(1–47) form. After its entry into lysosomes, EGF-(1–47) is subsequently degraded to small peptides and amino acids by one or more leupeptin-inhibitable proteases [8].

In *Xenopus* oocytes vitellogenin undergoes cleavage into large fragments within an endocytic compartment termed 'light yolk platelets' [24]. On the other hand, the rapid and complete degradation to TCA-soluble products of mannose-BSA has been reported within macrophage endosomes [1]. Within hepatic endosomes, insulin and glucagon also are totally degraded and transport to lysosomes is not required [4,6,13,14]. Endosomal degradation of glucagon arises from proteolytic modifications at the C-terminal (carboxypeptidase activity), internal (endopeptidase activity) and N-terminal (mainly tripeptidyl aminopeptidase activity) regions of the

Table 1. The substrates of endosomal proteolytic activities and their type of processing

Endosomal substrate	Responsible protease	Processing	Cleavage sites	References
Hormones				
Insulin	endosomal acidic insulinase (EAI)	complete	Y^{16}–L^{17}, G^{23}–F^{24}, F^{24}–F^{25}	[13]
Glucagon	cathepsins B, D and tripeptidyl aminopeptidase	complete	Q^3–G^4, F^6–T^7, T^7–S^8, D^9–Y^{10}, S^{11}–K^{12}, L^{14}–D^{15}, Q^{20}–D^{21}, F^{22}–V^{23}, V^{23}–Q^{24}, Q^{24}–W^{25}, W^{25}–L^{26}, L^{26}–M^{27}, M^{27}–N^{28}, N^{28}–T^{29}	[6]
PTH-(1–84)	cathepsin D	partial	F^{34}–V^{35}	[7]
Growth factors				
EGF	cathepsin B, carboxypeptidase B-like and trypsin-like	partial	L^{47}–K^{48}, K^{48}–W^{49}, L^{52}–R^{53}	[8]
Toxins				
Ricin A-chain	cathepsins B and D	partial	ND	[9,10]
Cholera	ND	partial	ND	[11]
Proteins of immune system				
Foreign antigens	cathepsins B and D	partial	ND	[40,43]
Invariant chain	cathepsins B and D	complete	ND	[51-54]
Antibodies	cathepsins B and D	partial	ND	[40]
Other proteins				
Apolipoprotein B-100	cathepsin D	partial		[18]
Vitellogenin	cathepsin D	partial		[24]
Mannose-BSA	cathepsin D	complete	ND	[1]
Tyramine-cellobiose asialofetuin	ND	partial	ND	[23]
Tyramine-cellobiose-BSA	cathepsin D	partial	ND	[23]

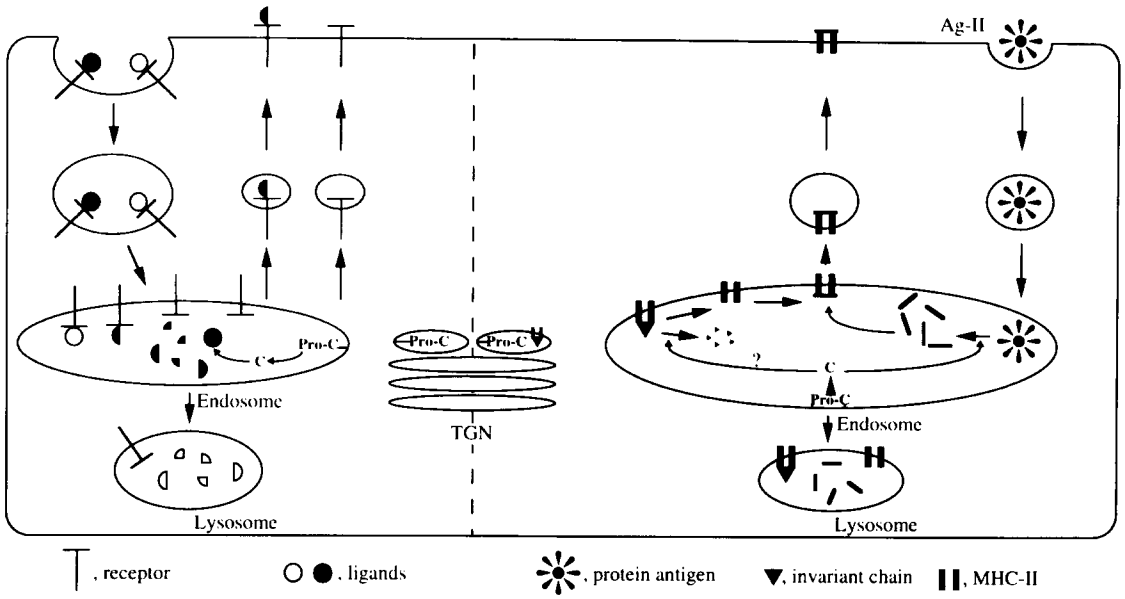

Fig. 1. The delivery and sorting of proteases and ligands into endosomes. Polypeptide hormones (left) and antigen proteins (right) are delivered from the cell surface into endocytic vesicles in which proteases are transported from intracellular sources such as the Golgi apparatus. TGN, *trans*-Golgi network; Pro-c, inactive procathepsins; c, active cathepsins; Ag-II, extracellular antigens which bind to class II MHC molecules.

hormone [3,4,6]; while endosomal insulin degradation appears to be largely effected by endopeptidase activity [13].

Endosomal proteolysis of internalized ligands regulates the intracellular fate of ligand and may be important both in terminating and in promoting intracellular signal transduction. The endosomal processing of insulin, EGF and glucagon produces fragments which are unable to bind to their respective receptors. Thus, one potential role of endosomal proteolysis of insulin and EGF may be to modulate (EGF) or terminate (insulin) receptor tyrosine kinase activation by limited (EGF) or complete (insulin) processing of the ligands [25]. In contrast, limited proteolysis of PTH-(1–84) to a smaller bioactive form PTH-(1–34) [7], and partial digestion of protein antigens by B-lymphocytes (see above) lead to the promotion rather than termination of signalling. This is effected by externalizing metabolites which serve functions (adenylate cyclase activation and immune response) different from that of the intact molecules. Also, inhibition of cathepsins B and D or neutralization of the acidic endosomal pH lead to the accumulation of A-chains of diphtheria and ricin toxins inside the cell and reduce toxin cytotoxicity [10,26]. Thus, the low endosomal pH and the restricted cleavages of plant and bacterial toxins upon delivery into endosomes appears to be critical in their translocation through the endosome membrane into the cytoplasm [10,11].

3. The nature of endosomal proteases

A number of studies have reported that lysosomal enzymes appear to reside permanently in the endosomal compartment (see Table 2). These biochemical and morphological studies have defined the thiol-dependent cysteine protease cathepsins B and H, and the pepstatin A-sensitive aspartic protease cathepsin D as responsible for the majority of proteolytic activities in endosomes [20]. Cathepsins B and D have been identified in endosomes of rabbit alveolar macrophages by biosynthetic studies and affinity chromatography purification [1,17], in hepatic endosomes by Western blotting and cross-linking procedures [6,18], and in multivesicular endosomes of macrophages [27] and B-lymphoblastoid cells [15] by immu-

Table 2. Properties of proteases identified in the endosomal apparatus of various cell types

Protease	Class	Optimum pH	Molecular weight	Proteolytic activity	Inactive proform in endosomes	cDNA cloned	References
EAI	metallo or cysteine	5.5	80?	endopeptidase	ND	no	[13,16]
Cathepsin B	cysteine	4 and 7	30	amino- and carboxypeptidase (pH 4) endopeptidase (pH 7)	yes	yes	[6,15,27,28]
Cathepsin D	aspartic	4	45	endopeptidase	yes	yes	[1,6,15,17,18,27,28]
Cathepsin H	cysteine	5	28	aminopeptidase	ND	yes	[20]
Carboxypeptidase B-like	ND	ND	ND	carboxypeptidase	ND	no	[8]
Trypsin-like	ND	ND	ND	endopeptidase	ND	no	[8]
Tripeptidyl aminopeptidase	ND	ND	ND	aminopeptidase	ND	no	[6]

nocytochemistry. The endosomal location of cathepsins B, D and H has been confirmed in several different cell types using synthetic fluorogenic substrates taken up by fluid-phase pinocytosis [28]. Both cathepsins B and D are active against a wide variety of substrates and catalyze the endosomal degradation of mannose-BSA, PTH, glucagon, ricin A-chain and vitellogenin. Leucyl-β-naphthyl amidase activity has also been detected in endosomes of HeLa cells [29].

However, other more poorly characterized endosomal proteases seem to have a more restricted substrate specificity (Tables 1 and 2). Thus, carboxypeptidase B-like and trypsin-like proteases are responsible for EGF processing within hepatocyte and fibroblast endosomes [8]. An endosomal acidic insulinase (EAI) has been partially purified using an affinity purification protocol [16]. EAI is a thiol endopeptidase with a partial metal ion requirement which differs from the previously extensively studied 'insulin-degrading enzyme' [16,30,31]. EAI may be restricted to cells highly enriched in insulin receptors. Thus, in contrast to Fao hepatoma cells, Chinese hamster ovary cells transfected with the human insulin receptor were unable to degrade insulin rapidly. Furthermore, the time course of degradation was identical to that of IGF-II and consistent with the delivery of internalized ligand to lysosomes [22].

The site of delivery of EAI, carboxypeptidase B-like and trypsin-like proteases into the endocytic pathway is unclear. Access to endosomes from the cell surface and/or by fusion of protease-containing vesicles directly from the Golgi apparatus are both possibilities. However, endosomal cathepsins B and D are transported to endosomes and lysosomes by the 215 kDa cation independent mannose 6-phosphate receptor (M6P-R), which acts to sort cathepsin transport at the level of the *trans*-Golgi network (TGN) [32]. After their delivery to endosomes as an inactive proform, the low endosomal pH promotes dissociation of procathepsins from M6P-R. M6P-R returns to the TGN while the endosomal procathepsins mature. Alternatively, secreted precursors may be taken into cells by receptor-mediated process and proteolytically cleaved to their mature form in endosomes [33]. The removal of propeptides from newly synthesized cathepsins requires proteolytic processing to attain full activity. Endosomal maturation of inactive procathepsins seems specifically to occur for only a few enzymes (such as procathepsins B and D), while other inactive proforms (such as procathepsin L) could require lysosomal delivery to undergo propeptide removal [6]. Some studies suggest that cathepsin D may autoactivate and may also activate other cathepsins such as cathepsin B [34].

The mechanism(s) by which cathepsins B and D are selectively retained within the endosomal compartment are not clear. Selective enrichment of these cathepsins in endosomes may be related to their association with endosomal membranes via undefined receptors distinct from the IGF-II/M6P-R [32]. These membrane associations may affect both inactive precursor (procathepsins B and D) and active mature enzymes (cathepsins B and D) [6,17]. The retention agents could be related to the procathepsin L receptor recently identified within NIH 3T3 cell lines [35]. Indeed, M6P receptor-independent membrane association has been reported within endosomes (or acidic prelysosomal vesicles) for procathepsins D and L in macrophages [17], in HepG2 cells [36], in mouse fibroblasts and in KNIH cells [35], as well as in Morris hepatoma 7777 cells for procathepsin C where the cation-independent M6P-R is absent [37]. Future studies will define the molecular mechanisms responsible for targeting and retaining proteases in endosomes.

4. Endosomal proteolysis of antigens for MHC-II presentation

Recent studies have established a critical function for endosomal proteases in processing protein antigens to small peptides which are subsequently bound to major histocompatibility complex (MHC) molecules for presentation at cell surface. MHC-I molecules primarily present peptides derived from cytosolic antigens that are transported into the endoplasmic reticulum (ER), such as viral antigens synthesized in infected cells [38]. Extracellular protein antigens bind to receptors such as immunoglobulins on B-cells and are subsequently taken up by receptor-mediated endocytosis into MHC-II-presenting cells [39]. Alternatively, exogenous protein antigens may be internalized into antigen-presenting cells by fluid phase endocytosis. Acid-optimal proteases partially process these internalized proteins within the endosomal compartment and generate degradation products [40]. Some degradation products which have a broad range of lengths, typically 12–24 residues [41], bind to MHC-II molecules and are thereby transported to the plasma membrane (Fig. 1). A similar scenario applies to antibodies and antigen-antibody complexes which bind to surface Fc receptors on immune cells and are rapidly internalized and subsequently proteolyzed within endocytic vesicles [40]. The unfolding and denaturation processes that are needed for peptide-antigen presentation are induced by the low endosomal pH. The importance of endosomal acidification and subsequent stimulation of acidic proteolytic activities in antigen processing and presentation is illustrated by the facts that: (i) cells defective in endosomal acidification have a reduced capacity to process foreign antigens [42]; (ii) in accordance with this, agents that raise endosomal pH, such as ammonium chloride and chloroquine, inhibit presentation of peptides to B-cells by MHC-II [43]; and (iii) the covalent coupling of antigen to transferrin leads to internalization of the complex into B-cells via the transferrin receptor, followed by antigen degradation within endosomes [44].

During intracellular transport, polymorphic αβ heterodimers of MHC-II glycoproteins associate with a type II transmembrane protein termed invariant chain (Ii) [39]. In the ER, Ii assembles with class II molecules to form a nonameric complex composed of three αβ dimers and an Ii trimer [45]. Ii displays various functions on MHC-II molecule transport by: (i) facilitating folding and assembly of class II molecules; (ii) preventing class II molecules from binding peptides in the ER; and (iii) directing the nonameric $(\alpha\beta Ii)_3$ complexes into a specialized endosomal compartment via targeting information in the Ii cytoplasmic tail [46]. The importance of Ii in the sorting of class II molecules is illustrated by the fact that, in the absence of Ii, class II molecules aggregate and associate with chaperone(s) in the ER [47]. Moreover, Ii 'knockout' mice display deficiency in cell surface expression of class II molecules and protein-antigen presentation [48].

Endosomal targeting signals have been mapped predominantly to a region in the N-terminal cytoplasmic domain that contains two dileucine-related signals and an acidic amino acid residue four or five residues N-terminal to each dileucine-related signal [46]. The precise location in the endosomal/

lysosomal pathway in which Ii accumulates and is proteolytically removed remains unclear. Morphological studies have shown that intracellular vesicles staining for Ii are enriched with endocytic markers, but also with late endosomal or lysosomal markers such as cation-independent M6P-R [49,50]. Once the $(\alpha\beta Ii)_3$ complexes arrive in endosomes, Ii is proteolytically degraded leading to the generation of N-terminal fragments, some of which remain transiently associated with class II molecules [51,52]. Complexes of $\alpha\beta$ with a set of 20–24 residue Ii fragments called CLIP (class II associated invariant chain peptide) have been identified as intermediates in class II molecule maturation [53]. The CLIP segment, which likely binds to the $\alpha\beta$ binding site in a way almost identical to that in which antigenic peptides bind MHC-II molecules [53], is necessary for in vivo Ii activity [54]. Proteolytic events also generate a 12 kDa N-terminal fragment of Ii which does not include the CLIP region of Ii and which remains associated with class II molecules for an extended period of time [51].

Thus, determination of the nature and class of endosomal proteases involved in antigen processing is complicated by the existence of two proteolytic steps: processing of Ii and that of antigen proteins. Moreover, it is still not known whether endocytic vesicles which process Ii correspond to the compartment in which antigenic peptide loading and proteolysis occur. Inhibitors of thiol-protease type enzymes, such as leupeptin, abolished degradation of Ii with subsequent inhibition of binding of peptides to MHC-II and expression of MHC-II on the cell surface [43]. Characterization of the relevant proteases with a panel of inhibitors has shown that acidic proteases play a central role in these two processing steps [43] with cathepsin B-like and cathepsin D-like activities of major importance [55]. Processing of specific antigens (such as myoglobin, ovalbumin and Ii) in vitro by purified cathepsins B and D generate peptides that can be presented to T-cells without further processing suggesting that these cysteine and aspartic proteolytic activities are likely the relevant proteases.

5. Conclusion

The endosomal system represents a cellular compartment located temporally and spatially between the plasma membrane and lysosomes. It is of regulatory significance in the selective proteolysis of polypeptide hormones and perhaps of major importance in peptide antigen presentation via the MHC class II pathway.

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