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Paul Saftig

Lysosomes

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Lysosomes

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LANDES BIOSCIENCE / EUREKAH.COM
GEORGETOWN, TEXAS
U.S.A.

SPRINGER SCIENCE+BUSINESS MEDIA
NEW YORK, NEW YORK
U.S.A.

LYSOSOMES

Medical Intelligence Unit

Landes Bioscience / Eurekah.com
Springer Science+Business Media, Inc.

ISBN: 0-387-25562-1 Printed on acid-free paper.

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Springer Science+Business Media, Inc., 233 Spring Street, New York, New York 10013, U.S.A.
http://www.springeronline.com

Please address all inquiries to the Publishers:
Landes Bioscience / Eurekah.com, 810 South Church Street, Georgetown, Texas 78626, U.S.A.
Phone: 512/ 863 7762; FAX: 512/ 863 0081
http://www.eurekah.com
http://www.landesbioscience.com

Printed in the United States of America.

9 8 7 6 5 4 3 2 1

Library of Congress Cataloging-in-Publication Data

Saftig, Paul.
Lysosomes / Paul Saftig.
p. ; cm. -- (Medical intelligence unit)
Includes bibliographical references and index.
ISBN 0-387-25562-1 (alk. paper)
1. Lysosomes. I. Title. II. Series: Medical intelligence unit
(Unnumbered : 2003)
[DNLM: 1. Lysosomes--enzymology. 2. Lysosomes--metabolism.
3. Biological Transport. 4. Lysosomal Storage Diseases--therapy.
QU 350 S128L 2005]
QH603.L9S24 2005
571.6'55--dc22

2005013404

Dedicated to Jane and Lynn ... and all patients suffering of diseases with an impaired lysosomal function.

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PREFACE

The lysosome is the cell's main digestive compartment into which many types of macromolecules are delivered for degradation. However, during the last ten years it has become evident that lysosomes have more complex functions than simply being the "waste basket" of the cell. Lysosomes can be involved in various cellular processes such as cholesterol homeostasis (Chapter 9), autophagy (Chapters 14 and 15), repair of the plasma membrane (Chapter 13), bone remodelling (Chapter 12), defence against pathogens, cell death, and signaling. More than 50 acid hydrolases have been identified which are involved in the ordered lysosomal degradation of a variety of proteins, lipids, carbohydrates, and nucleic acids (Chapters 5, 6 and 11). These hydrolases are enclosed by a limiting membrane containing a set of highly glycosylated lysosomal membrane proteins (Chapter 4). Lysosomal enzymes are also components of cell type-specific compartments referred to as lysosome-related organelles and include melanosomes, lytic granules, MHC class II compartments, platelet-dense granules, and synaptic-like microvesicles. Functional deficiencies of several lysosomal proteins give rise to the lysosomal storage diseases (Chapters 6, 7, 8 and 10).

The biogenesis of new lysosomes or lysosome-related organelles requires a continuous delivery of newly synthesized components. The targeting of acid hydrolases depends on the presence of mannose-6-phosphate residues that in turn are recognized by specific receptors, mediating the intracellular transport to an endosomal/prelysosomal compartment (Chapters 2 and 3).

The more I work with the lysosomal compartment the more I realize that lysosomes are not simply a dead end in the endocytotic pathway. The majority of physiological processes involve at least a transient encounter with this cellular compartment. A more detailed understanding of the functions of lysosomes will shed light on the molecular basis of pathological conditions in which the degradation and transport processes of intracellular molecules are affected. This is reflected by the exponentially increasing volume of literature dealing with the biology of the lysosome. This book will only be able to pinpoint some of the essentials of what has been published recently. The goal of this book is to introduce the major features of lysosomes at a level which should be useful for both interested students as well as researchers and clinicians in need of a broad background.

When I was asked to edit a book aiming to provide information about lysosomal functions, I was fascinated by the chance to meet and talk with some of the leading experts in the field, but also a little apprehensive about whether I would be able to cover both the novel findings and the more established lysosomal research. I am now glad that I had this opportunity to interact closely with many colleagues who have been extremely generous with their time and efforts. I am especially happy that this book has turned out to be a blend of contributions from young colleagues who are pushing

the lysosome community forward with new concepts and ideas as well as contributions from more experienced and well recognized experts in various aspects of lysosomal function.

Once again, I would like to express my gratitude to all contributors and to the editor who helped me to realize this project. Last but not least I would like to thank Kurt von Figura, my teacher and mentor in "lysosomal matters", for his continuous support and interest in my research. Without his advice I would have missed many interesting aspects of this intriguing organelle.

Paul Saftig

CHAPTER 1

History and Morphology of the Lysosome

Renate Lüllmann-Rauch*

Abstract

The lysosome is the cell's main digestive compartment to which all sorts of macromolecules are delivered for degradation. The structure of the lysosome is variable and depends on the cell type and the actual conditions. In terms of function and cytochemistry, the lysosome is identified by the following criteria: acid pH, hydrolases with acid pH optimum, specific highly glycosylated membrane-associated proteins, and the absence of the mannose-6-phosphate receptor. The purposes of the present chapter are (a) to give a short overview on the morphology of the lysosome/endosome system for readers who are nonexperts in this field; and (b) to briefly trail the tracks and approaches which, during the first decades after the discovery of the lysosome, led to the present concept, with particular reference to morphology.

Introduction

Lysosomes are membrane-delimited organelles which occur in all mammalian cells except red blood cells. Lysosomes are defined by functional rather than structural properties. They contain a high proton concentration (pH $\leq$ 5) and more than 40 hydrolases with a pH optimum below 6. Their limiting membrane is endowed with specific integral proteins including a vacuolar-type H^+-ATPase, several highly glycosylated proteins and various types of transporters as reviewed in Chapters 4, 7, and 9 of this volume. Lysosomes are engaged in the degradation of macromolecules delivered from the cell's own cytoplasm (autophagy, Chapters 14 and 15 of this volume) as well as materials taken up from the extracellular space (endocytosis). Depending on the cell type and the functional state, lysosomes can considerably vary in structure. Therefore it is not surprising that the existence of lysosomes was first realized solely on the basis of biochemical results. The term *lysosome* was coined by DeDuve five decades ago.[1] Only thereafter, ultrastructural and enzyme-cytochemical studies of Novikoff and coworkers[2,3] uncovered the morphologic identity of lysosomes. Expanding research in this field gradually revealed the lysosomes as being part of the highly dynamic endosome/lysosome system (Fig. 1), a collection of several categories of vesicular organelles which to a certain extent exchange membrane constituents and contents thus having overlapping properties. An important feature of lysosomes which serves to discriminate them from endosomes and other related vesicles is the absence of mannose-6-phosphate receptors.

Since the history of a scientific concept is appreciated particularly in the light of the present state of knowledge, the first part of this article briefly summarizes the basics of what is known today about the morphology of lysosomes including the morphological correlates of lysosome biogenesis. The second part provides a short historical review of the development of the lysosome concept during the first two to three decades following its original formulation.

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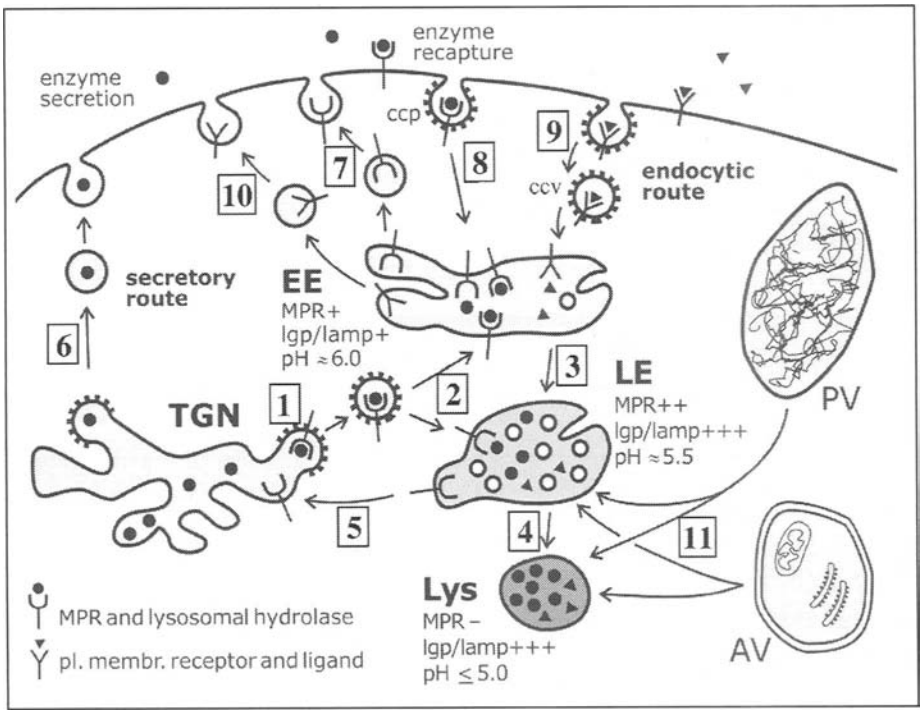

Figure 1. Diagrammatic summary of the endosome/lysosome system, with special reference to the biogenesis of lysosomes. After synthesis in the rER and modification in the Golgi apparatus (not shown) precursors of soluble lysosomal enzymes decorated with mannose-6-phosphate residues meet the mannose-6-phosphate receptor (MPR) in the trans Golgi network (TGN), are packaged (1) into clathrin-coated vesicles (ccv), and are transported (2) to late endosomes (LE) either directly or indirectly via early endosomes (EE) (3). The process of enzyme transfer (4) from the LE to the lysosome (Lys) is not fully elucidated yet; possibly LE matures to become Lys, or LE and Lys fuse to form a transient hybrid organelle (for further possibilities see text). The MPR is recycled (5) from the LE to the TGN, the lysosome is devoid of MPRs. A minor portion of the enzyme precursors gets into the secretory pathway (6) and is recaptured into clathrin-coated pits (ccp) by MPRs, which may be transferred (7) from the EE to the plasma membrane. Thus the enzyme precursors can reach the lysosome via the endocytic pathway (8) as do endocytic tracer molecules (9), whose receptors are recycled from the tubular extensions of the EE (10). Autophagic vacuoles (AV) and phagocytic vacuoles (PV) acquire lysosomal enzymes by fusion with lysosomes and/or LE (11) to become autolysosomes and phagolysosomes,[85] respectively. LE often resemble multivesicular bodies, i.e., they display invaginations of their membrane and internal vesicles budded off the invaginations (or representing cross sections of the invaginations). lgp/lamp, lysosomal membrane glycoproteins/lysosome-associated membrane proteins.

Morphology of Lysosomes

In terms of morphology, lysosomes are less clearly defined than other cell organelles such as mitochondria, peroxisomes or hormone storage granules. Yet, for normal cells of intact mammalian organisms lysosomes have so often been described in the literature, both ultrastructurally and cytochemically, that a combination of certain features may serve to identify lysosomes on the ultrastructural level. Lysosomes appear as cytoplasmic dense bodies, which are spheric, ovoid or occasinally tubular in shape, contain an amorphous osmiophilic dense matrix and are surrounded by a limiting membrane. Often lysosomes have a typical intracellular position, e.g., in hepatocytes near the bile canaliculus, in renal proximal tubule cells in the supranuclear cytoplasm, in cultured fibroblasts in the perinuclear cytoplasm. The size of lysosomes varies

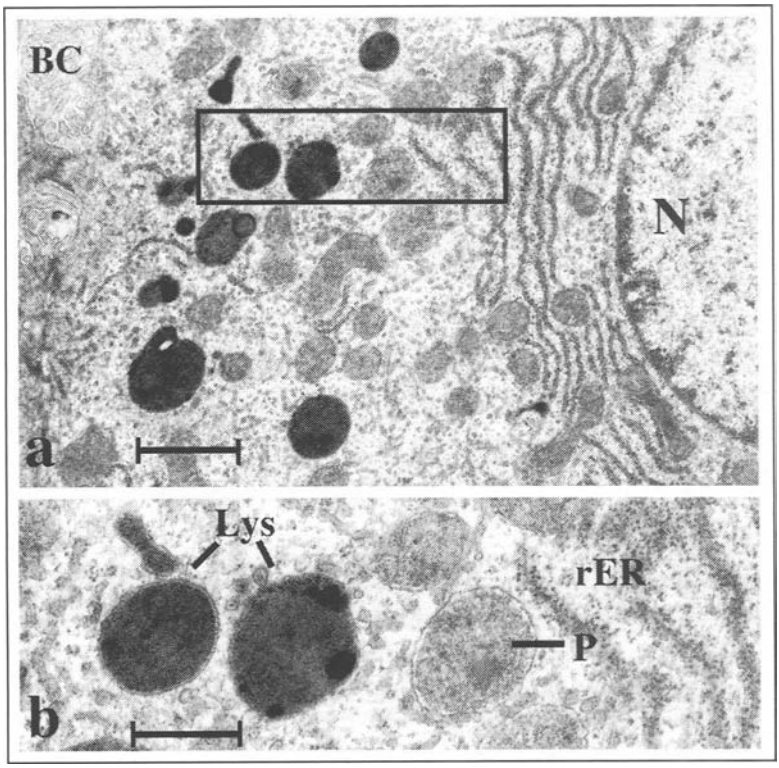

Figure 2. Lysosomes in a hepatocyte of a normal rat. a) The lysosomes appear as cytoplasmic dense bodies in close proximity to the bile canaliculus (BC). N, nucleus. b) Area marked in (a), shown at higher resolution. The limiting membrane and the amorphous dense matrix of the lysosomes (Lys) is seen. P, peroxisome. rER, rough endoplasmic reticulum. Bars represent 1 μm (a) and 0.5 μm (b).

between < 1 μm in many cell types as for example hepatocytes (Fig. 2) and neurons (Fig. 3) and several microns, e.g., in macrophages. On the light microscopic level (semithin epoxy resin sections), normal lysosomes—if visible at all—appear as intensely stained cytoplasmic bodies. Obviously, the diagnosis can only be tentative and requires verification by ultrastructural examination and/or enzyme-histochemical or immuno-histochemical demonstration of lysosomal constituents (enzymes or membrane proteins).

Different cell types show great quantitative differences regarding their equipment with lysosomes. In normal fibroblasts, hepatocytes or pituitary cells for example, the lysosomes usually contribute 0.5% or less to the cytoplasmic volume,[4,5] whereas in macrophages the fractional volume of lysosomes can be considerably larger.[6] The kidney may be taken as an example where the quantitative differences between cell types are immediately obvious (Fig. 4): The cells of the proximal tubules possess large numbers of lysosomes (corresponding to the high activity to endocytose and degrade proteins from the glomerular filtrate), whereas in the epithelia of the nephron segments downstream to the proximal tubules lysosomes are scarce.

The size and frequency of lysosomes can increase dramatically in any cell type, when the lysosomes accumulate nondegraded material. Such a condition can be induced by overloading the lysosomes with unphysiological substrates (e.g., saccharose,[7] polyvinylpyrrolidone, dextran), and by application of enzyme inhibitors (e.g., acarbose[8] inhibiting α-glucosidases or swainsonine[9,10] inhibiting mannosidases) or cationic amphiphilic drugs[11,12] and aminoglycoside

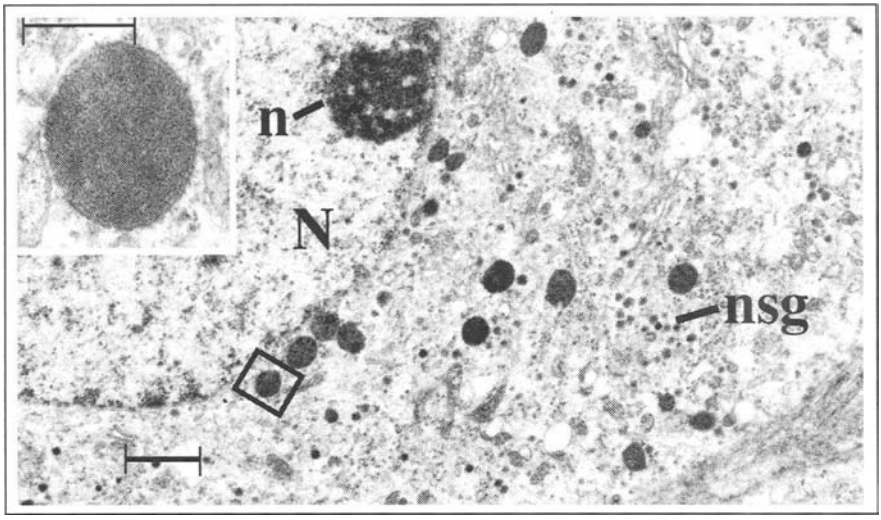

Figure 3. Lysosomes in a neurosecretory neuron from the hypothalamus of a normal rat. The lysosomes appear as dense cytoplasmic bodies. nsg, neurosecretory granules. N, nucleus. n, nucleolus. The *inset* shows one of the lysosomes at higher resolution. Bars represent 1 μm and 0.25 μm (inset), respectively.

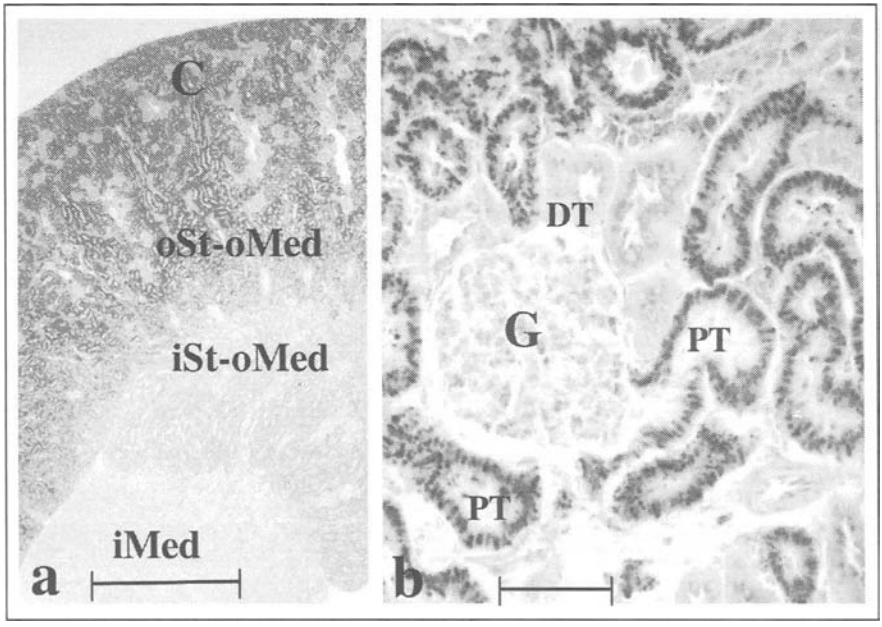

Figure 4. Kidney of a normal rat. Enzyme-histochemical demonstration of acid phosphatase.[86] The epithelium of the proximal tubules (PT) is rich of lysosomes, whereas in all downstream segments of the nephron (e.g., distal tubules, DT) and in glomerular cells (G) lysosomes are relatively scarce. Thus, intense staining is seen only in those regions which harbour PTs, i.e., cortex (C) and outer stripe of outer medulla (oSt-oMed). The inner stripe of outer medulla (iSt-oMed) and the inner medulla (iMed) are weakly stained. Bars represent 1 mm (a) and 50 μm (b).

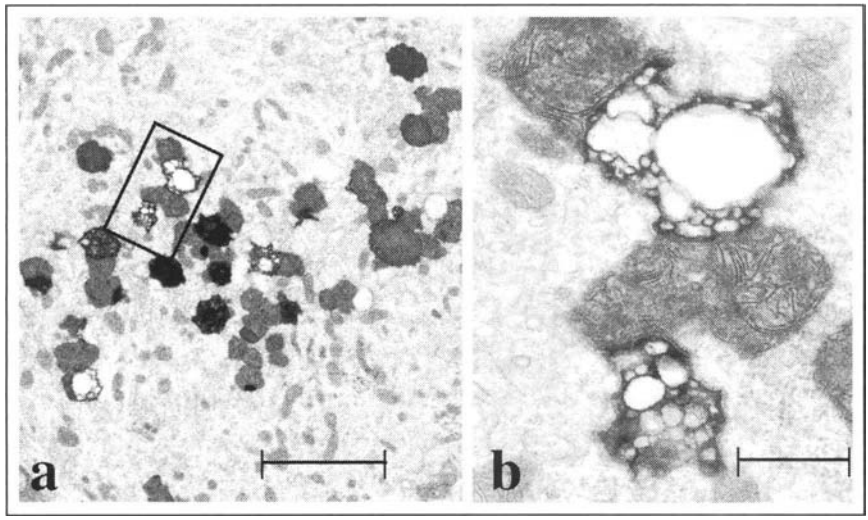

Figure 5. Lipofuscin granules in a dorsal root ganglion cell of an aged normal mouse (20 months). Several dense inclusions are seen in the cytoplasm, which are filled with polymorphous materials. Lipofuscin granules are generally regarded as telolysosomes, i.e., a subpopulation of lysosomes representing the terminal station for deposition of undigestible material which accumulates in long-lived cells. Bars represent 3 μm (a) and 0.5 μm (b).

antibiotics[13] which interfere with the intralysosomal digestion of certain substrates. Furthermore, an enormous augmentation of the lysosomal apparatus is typically seen in the inherited lysosomal storage diseases,[14-16] most of which are due to a genetically determined deficiency of a lysosomal enzyme (see Chapter 6 of this volume).

Contents of Lysosomes under Normal and Pathological Conditions

The structural heterogeneity of lysosomes is mainly a consequence of their function as digestive organelles. In macrophages of liver, lymphatic and hematopoietic tissues the lysosomes often are polymorphous and can contain remnants of undigestible or partly degraded materials. This is observed also in the retinal pigment epithelium[17] which constitutively engulfs and degrades fragments of the photoreceptor cell outer segments. Another example of lysosomes with polymorphous contents are lipofuscin particles, i.e., autofluorescent granules which are regarded as telolysosomes harbouring nondigestible ill-defined polymers of lipids complexed with proteins. Lipofuscin granules[18-20] typically accumulate in postmitotic and long-lived cells, for example neurons (Fig. 5), cardiomyocytes, hepatocytes or steroid-hormone producing cells.

The most dramatic accumulation of unusual contents is observed in inherited and induced lysosomal storage disorders. Thus, large amounts of intralysosomal glycogen particles are found in many cell types of individuals deficient in lysosomal acid α-glucosidase (glycogenosis type II, Pompe disease) (Fig. 6), and in experimental animals[8] treated with an inhibitor of this enzyme. In the majority of inherited lipidoses and drug-induced lipidosis, both of which are characterized by storage of polar lipids, the lysosomes are filled with multilamellated or paracrystalline materials reflecting the tendency of polar lipids to aggregate in highly regular arrays (Figs. 7, 9b). In a variety of lysosomal storage disorders characterized by accumulation of water-soluble substrates, for example mucopolysaccharidoses (storage of sulfated glycosaminoglycans) and mannosidoses (storage of low-molecular weight oligosaccharides containing mannose), the lysosomes in routine preparations appear as clear vacuoles (Figs. 8, 9c). This is

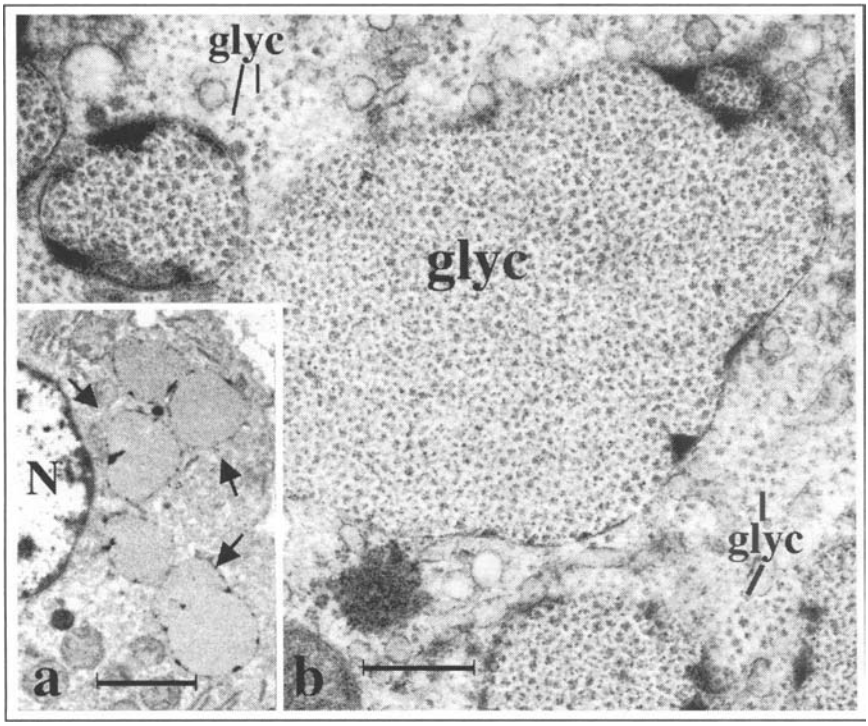

Figure 6. Lysosomal glycogen storage in a hepatocyte of a human fetus with Pompe disease (glycogenosis type II, deficiency for acid α-glucosidase). Glycogen rosettes (α particles) (glyc) are present in the cytoplasm (as observed also in normal hepatocytes) and in membrane-limited storage organelles (arrows in a). N, nucleus. Bars represent 2 μm (a) 0.5 μm (b).

due to the fact that the storage materials are not preserved by the usual fixation techniques and are therefore lost from the cells during tissue processing.

Biogenesis of Lysosomes

On the basis of the subcellular distribution of acid phosphatase as demostrated by enzyme cytochemistry, Novikoff and coworkers[21] proposed the concept of GERL (Golgi apparatus - endoplasmic reticulum - lysosome). It implied (a) that lysosomal enzymes, after biosynthesis in the rough endoplasmic reticulum (ER), are packaged into vesicles ("primary lysosomes") budding off from tubules which are continuous with the ER and intimately related to the Golgi apparatus, (b) that the vesicles are conveyed to preexisting lysosomes which have already been engaged in a digestive process ("secondary lysosomes"). This concept was later replaced by the concept of the *trans* Golgi network (TGN) as the common exit site for all products including lysosomal enzymes, secretory proteins and membrane proteins.[22,23] The most important modifications of the previous GERL concept were induced by data indicating that the intracellular transport of lysosomal enzymes is receptor-mediated and has to pass through a prelysosomal compartment (Fig. 1).

Separation of Lysosomal Enzymes from the Secretory Pathway

Most soluble lysosomal enzymes are synthesized as N-glycosylated precursors, the initial steps of biosynthesis are shared with the secretory proteins. The diversion of the lysosomal enzymes from the secretory pathway is dependent on the acquisition of the mannose-6-phosphate

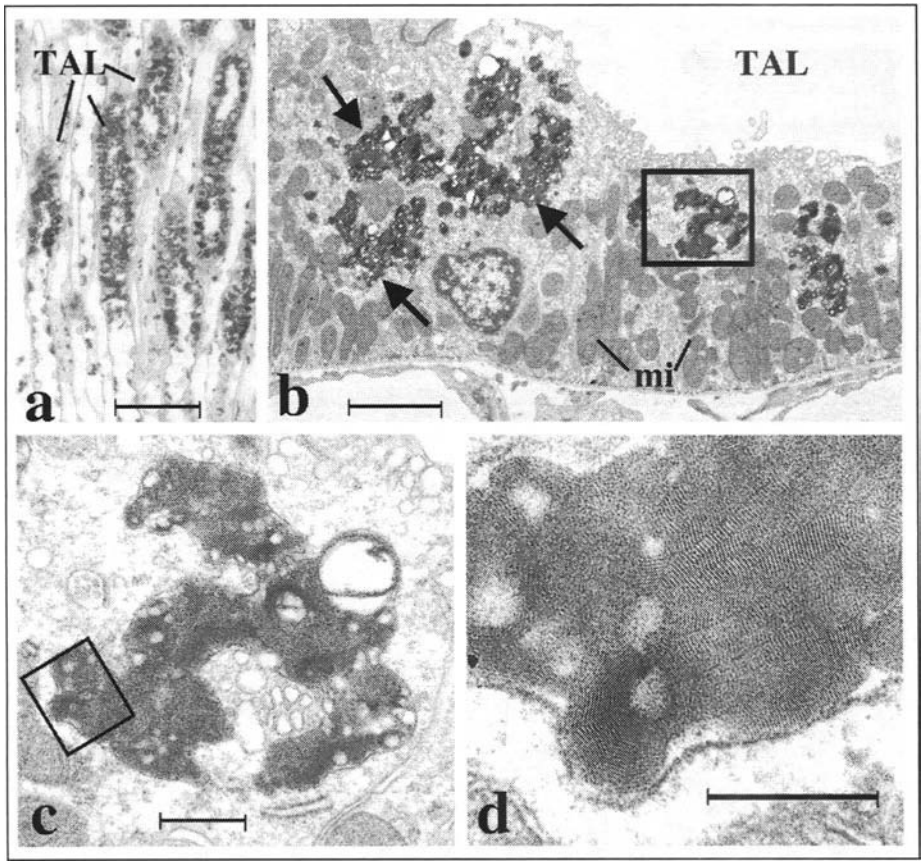

Figure 7. Lysosomal sulfatide storage in kidney of a mouse deficient in arylsulfatase A, representing an animal model for the metachromatic leukodystrophy (MLD) of humans.[87] a) Histochemical staining with alcian blue to detect sulfatide storage. All nephron segments shown in this photomicrograph are affected, particularly so the thick ascending limb (TAL) of Henle's loop, where normally the lysosomes are scarce. The micrograph shows the transition between inner medulla (bottom) and outer medulla. b) Ultrastructure of TAL epithelium. Bizzare-shaped inclusions are seen (arrows). mi, mitochondria. c) Lysosome marked in (b) shown at higher resolution. d) Portion from (c) shown at high resolution to show the limiting membrane and the fish-bone-like pattern, which is typical of intralysosomally accumulated sulfatides also in humans with MLD. Bars represent 50 μm (a), 4 μm (b), 1 μm (c), 0.5 μm (d).

(M6P) recognition marker; for review see reference 24. In the TGN the enzyme precursors meet transmembrane glycoproteins which recognize the M6P and bind the enzymes. Two distinct mannose-6-phosphate receptors (MPRs) have been characterized (for reviews see refs. 25, 26): The larger receptor is cation-independent (CI-MPR), whereas the smaller exhibits enhanced binding affinity in the presence of divalent cationes (cation-dependent, CD-MPR). The receptor-ligand complexes are sequestrated into clathrin-coated vesicles budding off from the TGN (see Chapters 2 and 3 of this volume). The vesicles are translocated and fuse with endosomes.

Endosomes

Endosomes can be roughly defined according to the temporal sequence in which they are reached by endocytic tracer molecules taken up from the extracellular space (Fig. 1); for reviews

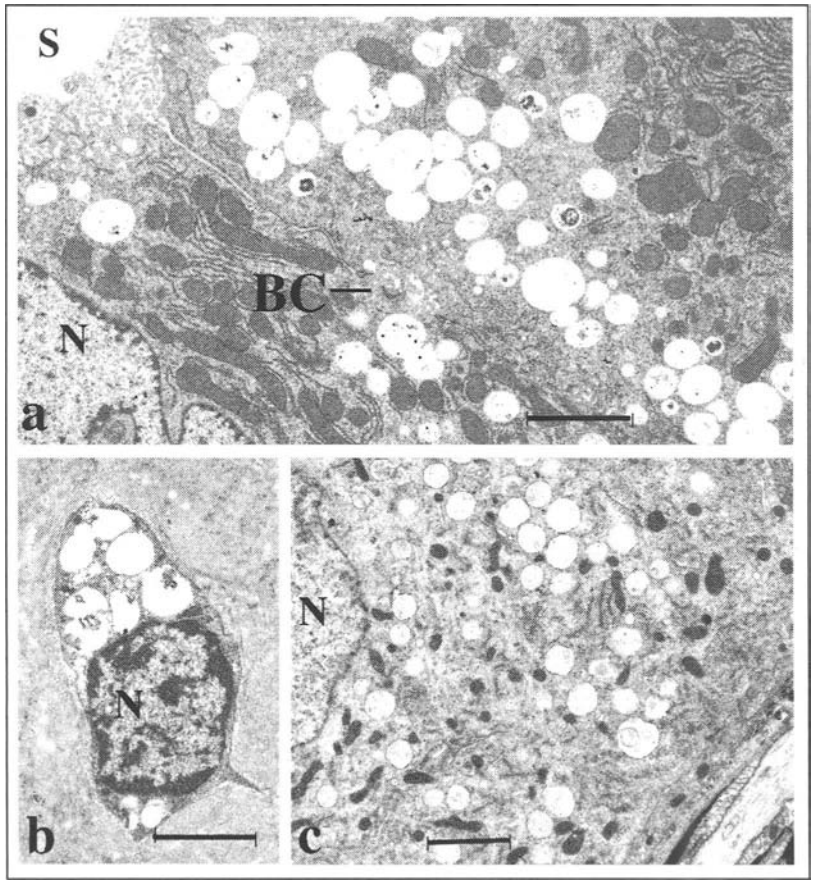

Figure 8. Lysosomes of mice deficient in α-mannosidase, which represent an animal model of the α-mannosidose of humans.[88] Hepatocyte (a), osteocyte (b), and vestibular ganglion cell (c). The lysosomes appear as empty vacuoles because the water-soluble storage material (mannose-containing oligosaccharides) are lost during tissue processing. BC, bile canaliculus. N, nucleus. S, sinusoid. Bars represent 3 μm (a) and 2 μm (b, c).

see references 27-29. After internalization by coated vesicles, the tracer appears in the early endosome (EE) within 1-5 min, in the late endosome (LE) within 10-15 min and accumulates in lysosomes after 30 min or later. EEs are less acidic (pH ≈6.0-6.2) than LEs (pH ≈5.5-6.0); for review see references 30, 31. EEs represents the compartment where the ligand-receptor complexes, if pH-sensitive, dissociate and from where the receptors are cycled back to the plasma membrane. The EE is often described to consist of two subcompartments (for reviews see refs. 32, 33): (a) the sorting endosome, i.e., electron-lucent vacuoles (diameter ≈0.5 μm) with tubular extensions; (b) the recycling endosome, i.e., tubules from where receptors are brought back to the plasma membrane and which are possibly formed by budding off from the sorting endosome. EEs can display a few internal vesicles which are formed presumably by budding off from invaginations of the limiting EE membrane. LEs are most probably generated by maturation of EEs. The internal vesicles (or membrane invaginations seen in cross section) increase in number as the EE matures to become an LE. Thus LEs often have the appearance of multivesicular bodies.

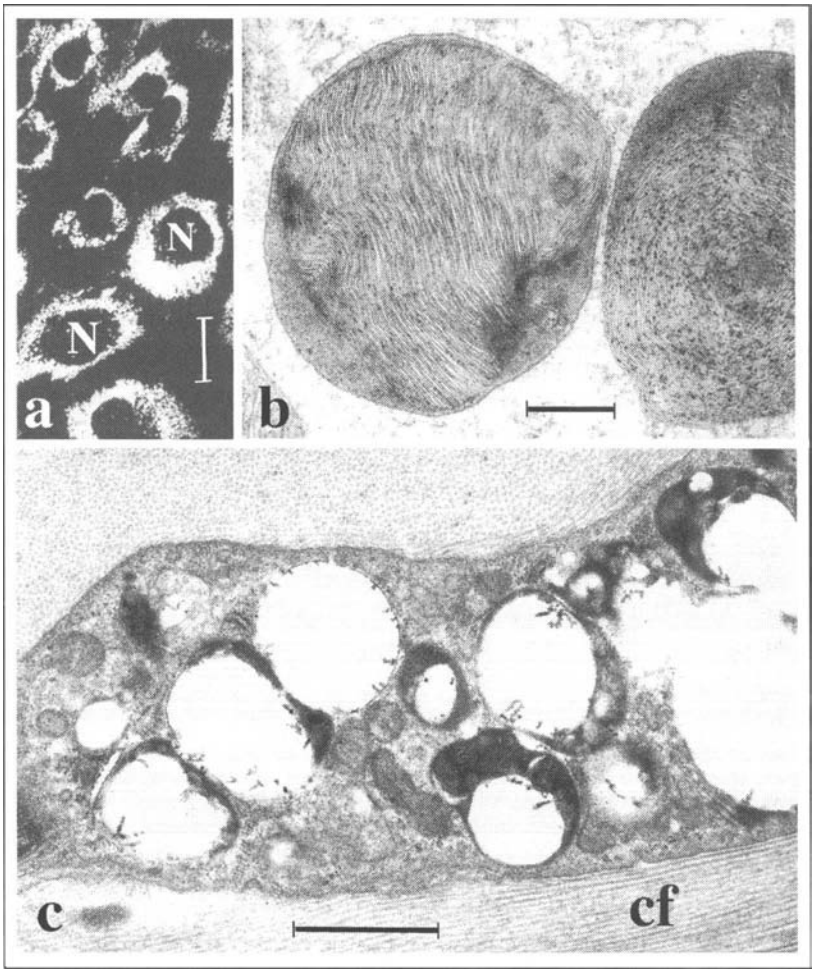

Figure 9. Induced lysosomal storage disorders as adverse drug effects. a) Cultured bovine corneal fibroblasts exposed to the antimalarial drug quinacrine (3 µM, 72 h).[49] Intralysosomal accumulation of the fluorescent drug. N, nucleus (unstained). b) Quinacrine-induced storage of polar lipids as indicated by the multilamellated material within lysosomes; choroid plexus epithelium[89] of rat after prolonged oral treatment with quinacrine, as an example of generalized drug-induced lipidosis.[11] c) Fibroblast from the cornea of a rat after prolonged oral treatment with the experimental anti-tumor drug tilorone,[90] as an example of generalized drug-induced mucopolysaccharidosis. The lysosomes appear as empty vacuoles because the water-soluble glycosaminoglycans, the storage of which has been demonstrated by histo- and biochemistry,[12] are lost during tissue processing. cf, collagen fibrils in longitudinal section and cross section (top). Bars represent 30 µm (a), 0.2 µm (b) and 1µm (c).

From the TGN to Lysosomes

It is not completely clear where the transport of lysosomal enzyme precursors merges with the endocytic pathway. The TGN-derived transport vesicles may deliver their cargo to the LEs directly or via the EEs. In any case, the LE is the main compartment where the pH-dependent MPR-enzyme binding dissociates. The receptors are retrieved into small vesicles, shuttled back to the TGN and become available for another round of enzyme tranfer (receptor recycling).

The last steps of enzyme maturation (dephosphorylation and trimming of the oligosaccharides and proteolytic processing) may be initiated in the LE and completed after arrival in the lysosomes. The way from the LE to the lysosome is not yet fully elucidated; the following possibilities have been proposed (for reviews see refs. 27, 34-36): (a) The LE itself or major portions of it may undergo further maturation to become lysosomes; (b) the lysosomal enzymes and lysosomal membrane proteins may be packaged into transport vesicles which fuse with preexisting lysosomes; (c) the LE and preexisting lysosomes may transiently fuse with each other ("kiss-and run")[37] to exchange contents, or (d) they may fuse to form transient hybrid organelles, followed by reformation of dense-core lysosomes. In any case, the lysosomes are, in contrast to the prelysosomal compartments, devoid of MPR. Under steady-state conditions, the MPRs are present in several populations of endosomes, in the Golgi apparatus, coated vesicles and on the cell surface, but consistently absent from lysosomes; for reviews see references 24, 27, 38.

Enzyme Secretion

The sorting mechanisms in the TGN are not fully efficient. Minor amounts of lysosomal enzyme precursors get into the secretory route, are released by exocytosis and recaptured by receptor-mediated endocytosis with the CI-MPR being responsible for the recapture. Cells of the monocyte/macrophage lineage additionally possess a mannose receptor which may be involved in the recapture of lysosomal enzymes.[39,40] The phenomenon of recapture is the basis for the "cross-correction" (see later) of genetically determined lysosomal storage and thus one of the mechanisms by which inherited lysosomal storage diseases are hoped to be treated efficiently (either by enzyme replacement or by transplantation of stem cells as founders of a competent cell population which serves as enzyme donor) (see Chapter 10 of this volume).

Alternative Sorting Mechanisms

In addition to the MPR-mediated trafficking of soluble lysosomal enzymes there may be additional or alternative mechanisms.This is concluded e.g., from findings on MPR knockout-mice[41] and from data on the human I-cell disease,[42] where the genetically determined lack of the M6P-recognition marker leads to secretion of the lysosomal enzymes from some but not from all cell types. Furtheremore, the MRP-dependent mechanism does not apply to membrane-associated lysosomal enzymes such as for example acid phosphatase, which is synthesized as a transmembrane protein and processed to a soluble enzyme only after arrival in the late endosome or lysosome. For a recent review on alternative mechanisms see reference 43.

Lysosomal Drug Accumulation and Drug-Induced Lysosomal Storage Disorders

A remarkable property of the lysosomes and associated acid compartments is the ability to accumulate weakly basic amines such as ammonia and chloroquine ("lysosomotropic" or "acidotropic" agents),[44] which is thought to be due to intralysosomal protonation of the bases and thus trapping within the acidic organelles. Using fluorescent lysosomotropic agents such as acridine orange or quinacrine, this principle can be used to visualize lysosomes in living cells (Fig. 9a). If such agents are applied at sufficiently high concentrations, they raise the pH in the acidic compartments,[45] interfere with the dissociation of the MPR and its ligand and thus with recycling of free MPRs. As a consequence, major proportions of the enzyme precursors get into the secretory route,[46,47] the cell is deprived of lysosomal enzymes, and all sorts of undigested macromolecules accumulate in the lysosomes. Apart from being useful tools for persuading cultured cells to secrete their lysosomal enzyme precursors, many of these weak bases, if possessing an aromatic ring system and thus being cationic amphiphilic in their charged form, can induce dramatic lysosomal storage of polar lipids and/or sulfated glycosaminoglycans in vivo and in vitro - notably at concentrations well below those enhancing enzyme secretion.[48,49] The intralysosomally accumulated drugs form fairly stable complexes

with polar lipids and/or sulfated glycosaminoglycans and thereby interfere with the digestion of the respective substrates. This results in adverse drug actions, which can be elicited not only in experimental animals but occur also in patients treated e.g., with the antiarrhythmic drugs amiodarone and perhexiline or the antimalarial and antirheumatic drug chloroquine (for review see refs. 11, 12, 50).

History of the Lysosome Concept

The route to the discovery of lysosomes is one of numerous examples in the history of science, where researchers while dealing with a scientific problem of their choice happened to encounter a side observation, which was curiously taken up and eventually led to a new biological concept.[51]

In the context of their primary interest in the effects of insulin on isolated liver tissue, DeDuve's group aimed at characterizing the glucose-6-phosphatase including its discrimination from an unspecific acid phosphatase activity. After having used aqueous liver extracts initially, they changed to cell fractionation, with isotonic sucrose solution serving as suspension medium. They made use of the differential centrifugation technique which had previously proven helpful in isolating mitochondria and identifying them as the exclusive cell organelles with cytochrome oxidase activity.[52] During their work, which led to the identification of glucose-6-phosphatase as a marker enzyme of the microsome fraction, DeDuve and coworkers observed that the acid phosphatase, which was distributed to several cell fractions, displayed "structure-linked latency": By diligent analysis of what initially appeared as an experimental failure, they found that the enzyme showed unexpectedly low activity in the homogenate and cell fractions unless the materials were treated by freezing and thawing or osmotic shock prior to the enzyme assay. This led to the conclusion that the enzyme might be enclosed within membrane-limited organelles thus being unaccessible for the substrate as long as the surrounding membranes were intact. Several other acid hydrolases (cathepsin, β-glucuronidase, ribonuclease and desoxyribonuclease) also showed latency and were codistributed with acid phosphatase to the same cell fractions. These observations led to the proposal that there must be a distinct group of "granules" or "lytic bodies" which DeDuve and coworkers in 1955 called *lysosomes.*[1] Thereafter the "granules" as isolated from liver tissue homogenates were shown by electron microscopy[2] to be membrane-bound, and they were demonstrated by enzyme cytochemistry[3] to correspond to the cytoplasmic "dense bodies" (pericanalicular or peribiliary bodies) usually present in hepatocytes (Fig. 2).

Soon after the discovery of lysosomes in hepatocytes this organelle was identified also in the epithelium of renal proximal tubules[53,54] which long before had been known to absorb various high molecular weight substances from the glomerular filtrate. This opened the way to unveil the physiological link between lysosomes and endocytosis;[55,56] for further historical reviews see references 57, 58. During this period also the relationship between lysosomes and autophagy was suggested[59] and demonstrated.[60]

Lysosomal Storage Diseases

The biological and medical importance of the lysosome concept was further underscored by the discovery in 1963 that one of the glycogen storage diseases (glycogenosis type II, Pompe disease) was due to the absence of a lysosomal enzyme (acid α-glucosidase).[61,62] The biochemical observations were confirmed by the ultrastructural finding that large amounts of glycogen particles were enclosed in membrane-bound organelles[63] (Fig. 6) rather than deposited in the cytosol as observed in all other types of glycogen storage diseases. These early observations led to the concept of *lysosomal storage diseases* and thus to an understanding of the mechanisms underlying several devastating storage diseases which had been described by clinicians decades before; for review see reference 14. Pompe disease was the first of the lysosomal storage disorders where attempts—without success though—of enzyme replacement (using a fungal extract with amylase and maltase activity) were performed.[63,64]

Biogenesis of Lysosomes

Concerning the biosynthesis of lysosomal enzymes and their delivery to the lysosomes, important impulses came from the work of Neufeld and her group.[65] They coined the term *cross-correction.* It refers to the original observation in 1969 that cocultured fibroblasts derived from two patients with different lysosomal storage disorders mutually corrected each other (in the original report Hurler and Hunter syndroms, two types of mucopolysaccharidoses which are characterized by lysosomal storage of sulfated glycosaminoglycans). The same was found when Hurler cells were cocultured with normal cells. This effect was explained by unknown "correction factors" which were proposed to be secreted into the medium by donor cells and taken up by the recipient cells and accelerated the degradation of the intralysosomal storage material.[66] Soon it became clear that the "Hurler corrective factor" was, in fact, the missing enzyme (α-L-iduronidase)[67] and that the enzyme was taken up into the deficient cells by a highly selective process requiring a recognition marker on the enzyme protein (involving a carbohydrate sensitive to oxidation by periodate).[68] Furthermore, another soluble lysosomal enzyme (β-glucuronidase) was found to exhibit heterogeneity regarding electrical charge and uptake, with the "high-uptake" form being more acidic than the "low-uptake" form of the enzyme.[69]

In 1977, the recognition marker was found to be a mannose-6-phosphate (M6P) residue; this was concluded from the findings that enzyme uptake (β-glucuronidase) was inhibited competetively by certain sugar phosphates and abolished by pretreatment of the enzyme with alkaline phosphatase.[70] The kinetics of enzyme binding (α-L-iduronidase) onto the cell surface were characterized, while the receptor was still unknown, and it was shown that the binding sites on the cell surface were regenerated at a fast rate,[71] which reflects receptor recycling as we now know.

These findings had initially led to the *"secretion-recapture hypothesis"* implying that newly synthesized lysosomal enzymes are normally secreted in a "high-uptake" form (i.e., possessing the M6P residue as a recognition marker) and then recaptured by the cells. This seemed also to explain the subcellular mechanism underlying the I-cell disease: It is a lysosomal storage disorder where cells, at least fibroblasts, are deprived of lysosomal enzymes because the latter lack the recognition marker and are released into the medium without being recaptured.[42] In 1978 the secretion-recapture hypothesis had to be modified, however, since normal cultured fibroblasts could not be deprived of lysosomal enzyme (β-N-acetylglucosaminidase) under conditions which interfered with enzyme uptake from the medium (e.g., effective extracellular concentrations of M6P); thus an alternative hypothesis was proposed:[72] Only a minor portion of newly synthesized enzyme might be secreted, while most might be bound to a yet hypothetical receptor within the vesicles budding from the Golgi apparatus, delivered to the plasma membrane without ever dissociating from the receptor and then conveyed to the lysosomes by the endocytic pathway.

Subsequently (1980) this hypothetical receptor, present not only on the cell surface but also on intracellular membranes, was shown to bind the enzyme (β-glucuronidase)[73] at neutral pH but releasing it at pH < 6 and to recycle at fast rate between the plasma membrane and the intracellular space.[46] The vast majority of M6P-binding sites was shown to be associated with intracellular membrane-limited organelles rather than with the plasma membrane.[74] The collective data led to the hypothesis that the receptor acts as a vehicle for the direct intracellular transport of newly synthesized lysosomal enzymes from the Golgi apparatus towards the lysosome and that the acid pH in the lysosome be decisive for dissociation of the enzyme-receptor complex und thus for reutilization of the receptor; for review see reference 75. In 1981 a mannose-6-phosphate receptor with an apparent molecular weight of 210 kDa (now known as the CI-MPR) was isolated and partially characterized,[76] the CD-MPR was described in 1985.[77]

By that time the intracellular route of the enzyme transfer and the site of receptor sorting were yet unsettled. Elucidation was greatly promoted by the concept of *receptor-mediated endocytosis* (for review see ref. 78) and the introduction of the *endosome* or receptosome[79] as an

acidic compartment intermediary between the clathrin-coated vesicles budding off from the plasma membrane and the lysosome; for reviews see references 30, 31, 80, 81. The important function of this organelle as a compartment of uncoupling receptor and ligand (CURL)[82] was demonstrated by immuno-electron microscopy; the tubular extensions of the CURL were proposed as the site from where receptors are recycled to the plasma membrane. Soon it was shown that the endosomal compartment could be devided into several subcompartments,[83] with the early endosome being the station responsible for recycling of plasma membrane receptors. In 1988 the late endosome was described to be of predominant importance for recycling of the MPR to the TGN.[84]

Concluding Remarks

The development of the lysosome concept is an exciting chapter of biosciences, where combined techniques of biochemistry, morphology, cell biology and more recently molecular biology have contributed and still contribute to the understanding of a functional system which is equally important for the homeostasis of the normal cell household and for pathological processes.

Acknowledgements

I am thankful to Dagmar Niemeier for excellent technical assistance and to Heidi Waluk and Clemens Franke for expert assistance with the art work. The EM illustrations presented in this review originate from studies which were supported by the Deutsche Forschungsgemeinschaft (Lu 172).

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CHAPTER 2

Transport of Lysosomal Enzymes

Stephan Storch* and Thomas Braulke

More than 50 acid hydrolases involved in the ordered lysosomal degradation of a variety of proteins, lipids, carbohydrates, and nucleic acids have been identified. The hydrolases are enclosed by a membrane containing a set of highly glycosylated lysosomal membrane proteins. Lysosomal enzymes are also components of cell type-specific compartments referred to as lysosome-related organelles which include melanosomes, lytic granules, MHC class II compartments, platelet-dense granules, and synaptic-like microvesicles.[1] The biogenesis of new lysosomes or lysosome-related organelles requires a continuous substitution with newly synthesized components. The targeting of acid hydrolases depends on the presence of mannose 6-phosphate (M6P) residues that are recognized by specific receptors mediating the intracellular transport to an endosomal/ prelysosomal compartment. The acidification of endosomes, lysosomes, and lysosome-related organelles facilitates not only the dissociation of the receptor-ligand complexes, but also the proteolytic processing required for the enzymatic activation of several hydrolases as well as the denaturation of proteins as prerequisite for lysosomal proteolysis.

Synthesis and Modifications of Soluble Lysosomal Proteins

Lysosomal hydrolases are synthesized with an N-terminal sequence of 20-25 amino acids recognized by the signal recognition particle which enable the nascent polypeptides to be translocated across the membrane of the endoplasmic reticulum (ER, Fig. 1, step 1). After removal of the signal peptide by a signal peptidase, preformed oligosaccharides are transferred to certain asparagine residues which are part of an N-glycosylation consensus motif Asn-X-Thr/Ser.[2] Typically, the oligosaccharides are composed of 3 glucose, 9 mannose and 2 N-acetylglucosamine residues ($Glc_3Man_9GlcNac_2$). The oligosaccharides undergo extensive processing before completion of translation by removal of the outmost glucose residues catalyzed by glucosidase I and II. Monoglycosylated core glycans of the newly synthesized polypeptide then bind to the molecular chaperone calnexin until the protein is properly folded.[3] Furthermore, all members of the sulfatase family responsible for the hydrolysis of sulfate esters from sulfated mono- and polysaccharides, glycolipids and hydroxysteroids, are modified in the ER generating a Cα-formylglycine (FGly) residue. The oxidation of a highly conserved cysteine occurs when the protein has not yet been folded and is catalyzed by the FGly-generating enzyme (FGE).[4] Mutations in the gene encoding the human FGE result in the appearance of multiple lysosomal sulfatases lacking enzymatic activity.[5,6] Moreover, aspartylglucosaminidase, a lysosomal enzyme that hydrolyzes the amide bond between asparagines and N-acetylglucosamine, has been reported to be rapidly activated in the ER by a proteolytic cleavage of the inactive precursor into α- and β-subunits which is triggered by the dimerization of the two precursor molecules.[7]

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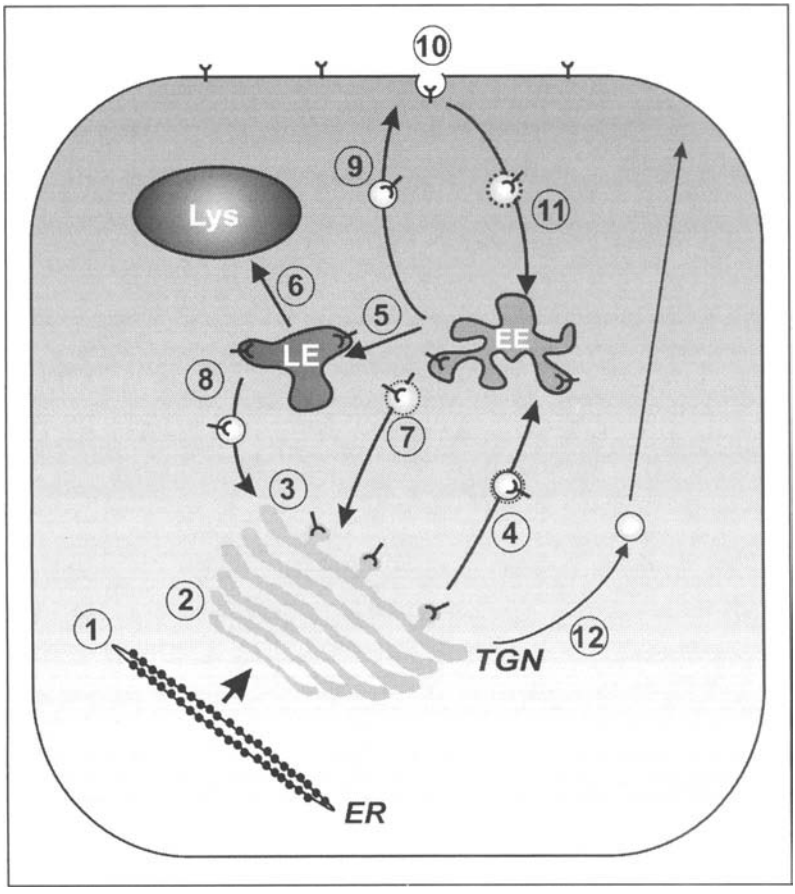

Figure 1. Model for the intracellular transport of MPR and proteins to lysosomes. Soluble lysosomal enzymes are synthesized and translocated into the lumen of the ER (step 1). In the Golgi (step 2) the enzymes are equipped with the M6P recognition marker (step 3) followed by binding to MPRs. The receptor-ligand complexes are transported to the early endosomal compartment (EE; step 4). Due to the low pH the receptor-ligand complexes dissociate and the lysosomal enzymes are delivered to the lysosome (Lys, steps 5 and 6). The MPRs recycle either back to the TGN (steps 7 and 8) or to the plasma membrane (step 9). Exogenous M6P-containing proteins can be internalized by CI-MPR (step 10) and are transported to lysosomes along the endocytic pathway (step 11). Lysosomal enzymes escaping binding to MPR in the TGN are secreted (step 12).

Formation of Mannose 6-Phosphate Recognition Marker

Upon arrival in the Golgi, the oligosaccharide chains of lysosomal enzymes are further trimmed and modified by the addition of complex sugar residues (galactose, fucose, and sialic acid), sulphate groups, and by the formation of the M6P recognition marker. This marker is generated by the sequential action of two enzymes.[8] In the first step, GlcNac-1-phosphate is added to the C6-hydroxyl group of selected mannoses on high mannose-type oligosaccharides by the enzyme UDP-*N*-acetylglucosamine: lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase (phosphotransferase) (Fig. 1, step 2). The purified bovine phosphotransferase is a 540 kDa heterohexameric complex composed of two disulfide-linked homodimers of 166 and 51 kDa

subunits and two noncovalently associated 56 kDa subunits ($\alpha_2\beta_2\gamma_2$).[9,10] The phosphotransferase γ-subunit has been shown to form cysteine-linked dimers[11] and contains two N-linked oligosaccharides (S. Tiede and T. Braulke, unpublished results). It has been proposed that the α and β-subunits harbour phosphotransferase activity and the γ-subunit functions in recognition of lysosomal enzymes.[12] Studies with chimeric proteins between cathepsin D and pepsinogen, mutant cathepsin L and aspartylglucosaminidase, and blocking antibodies against various epitopes on arylsulfatase A suggest that 2-3 lysine residues, separated by a distance of ~ 34 Å residing in distinct regions of the enzymes, are critical phosphorylation signals.[13-17] In the second step, N-acetylglucosamine residues are removed by an *N*-acetylglucosamine-1-phosphodiester α-*N*-acetylglucosaminidase (UCE; uncovering enzyme) exposing the M6P recognition marker (Fig. 1, step 3). The human UCE is a type I membrane spanning glycoprotein of 515 amino acids with a transmembrane domain and a cytoplasmic tail of 41 amino acids.[18] UCE is mainly localized in the TGN and constitutively cycles via the plasma membrane.[19] UCE is synthesized as an inactive proenzyme which is activated in the TGN by the cleavage of an N-terminal pro-peptide catalyzed by the endoprotease furin.[20] The bovine UCE exists as a tetramer composed of two disulfide-linked homodimers with molecular masses of 68 kDa for each monomer.[21] Following the uncovering of the M6P marker, lysosomal enzymes can be recognized by mannose 6-phosphate receptors (MPRs).

Mannose 6-Phosphate Receptors

In mammalian cells two MPR exist, one is a 46 kDa cation-dependent (CD-MPR, MPR46) and the other an 300 kDa cation-independent (CI-MPR, MPR300).[22] Both MPRs are type I membrane glycoproteins that differ in their developmental expression pattern, subcellular localization, quarternary structure, half life of receptor protein, ligand binding properties and functions.[23] The MPRs bind mono- or diphosphorylated oligosaccharides with a K_d of 2×10^{-7} -2×10^{-9} M per mole of receptor. Subsequently the receptor-ligand complexes exit from the TGN in clathrin-coated vesicles and fuse with membranes of the early or late endosomal compartment (Fig. 1, steps 4, 5). Due to the low pH, the lysosomal enzymes dissociate and are delivered to lysosomes (Fig. 1, step 6) and the MPRs recycle back to the TGN to mediate further rounds of transport (Fig. 1, steps 7, 8).[22] Small amounts of both MPRs are localized at the plasma membrane (Fig. 1, step 10) but only the CI-MPR is capable of binding and internalizing M6P-containing lysosomal enzymes (Fig. 1, step 11).

CI-MPR

The bovine CI-MPR comprises a short NH_2-terminal signal sequence, a 2269 residue extracytoplasmic domain, a single transmembrane domain and a 167 residue cytoplasmic domain. The extracytoplasmic domain contains 15 repeating segments of approximately 147 amino acids each sharing 16-38% of identical residues.[24] The two M6P-binding sites have been mapped to luminal domains 3 and 9 and residues Arg435 and Arg1334 being essential for M6P binding.[25,26] While domain 9 alone folds into a high affinity carbohydrate recognition site (K_d = 0.3 nM) domain 3 displays only low affinity (K_d ~ 500 nM) suggesting an importance of additional residues in adjacent domains 1 and/or 2.[25] CI-MPR also binds the nonglycosylated insulin-like growth factor II (IGF II).[27] The IGF II binding site has been mapped to repeat 11 in the extracellular domain of the CI-MPR.[28,29] Binding of IGF II inhibits binding of lysosomal enzymes and their internalization, whereas the sorting and the transport of newly synthesized lysosomal enzymes are not affected by the overexpression of IGF II.[27] The CI-MPR forms dimers induced by the binding of multivalent M6P-ligands but not by IGF II or M6P[30,31] resulting in an increased receptor internalization rate at the plasma membrane.[32] Besides soluble lysosomal enzymes, the CI-MPR binds other M6P-containing nonlysosomal proteins such as TGF-β1-precursor, proliferin, granzyme B, as well as other classes of ligands like retinoic acid, the urokinase-type (plasminogen activator) receptor, and plasminogen.[27,33]

CD-MPR

The 279 amino acid precursor of the bovine CD-MPR consists of a signal sequence, a 159-residue extracytoplasmic domain, a transmembrane region, and a 67 residue cytoplasmic tail.[34] The extracytoplasmic domain of the CD-MPR is similar to the repeating units of the CI-MPR and contains a single M6P-binding site. The CD-MPR binds 1 mol M6P or 0.5 mol diphosphorylated oligosaccharides per mole of monomeric receptor.[35]

The structural basis for the ligand binding properties of the CD-MPR was established by determining the three dimensional structure of both the complexed[36,37] and the uncomplexed[38] extracellular domain of the receptor. The CD-MPR complexed with M6P crystallizes as a dimer with each monomer consisting of an N-terminal α-helix followed by nine primarily antiparallel β-strands.[36] The M6P binding cavity is composed of two loops between β-strands 6 and 7 and between 8 and 9 that are linked by a disulfide bridge between residues Cys106 and Cys141. The residues His105 and Arg111, shown to be important for M6P-recognition,[39] are located within the ligand-binding site. The distance of 40 Å between the 2 ligand-binding sites of the dimer provides a structural basis for the observed differences in binding affinity exhibited by the CI-MPR toward various lysosomal enzymes. Crystal structures of the extracellular domain of the bovine CD-MPR complexed to pentamannosyl-phosphate revealed that the binding site of the receptor encompasses the phosphate group plus three of the five mannose rings of the ligand.[37] The binding properties of ligands to the extracellular domain of the CD-MPR are pH-dependent. The CD-MPR binds to lysosomal enzymes optimally in the Golgi (pH 6.5), releases its ligands in the acidic enviroment of the endosomal compartment (pH <6.0), and fails to interact with lysosomal enzymes at the plasma membrane (pH 7.4). Based on the crystal structure of the ligand-free extracytoplasmic CD-MPR domain it was suggested that the pKα of Glu133 is responsible for the release of lysosomal enzymes in the late endosomal compartment. On the other hand, the pKα value of M6P and His105 of the CD-MPR appear to be responsible for the inability to bind lysosomal enzymes at the cell surface.[38]

Signal Structure-Dependent Trafficking of MPRs

The MPRs are localized in the TGN, early endosomes, recycling endosomes, late endosomes and at the plasma membrane, but they are not found in lysosomes. Both receptors cycle constitutively between these compartments directed by signal structures located in their cytosolic tails. The efficient lysosomal enzyme sorting in the Golgi mediated by the CI-MPR depends on an acidic cluster/ dileucine-based sorting motif near the carboxyl terminus.[40-42] The rapid internalization of the CI-MPR from the plasma membrane requires a tyrosine-based sorting motif (Fig. 1, step 10).[43]

The cytoplasmic tail of the CD-MPR contains several signal structures important for anterograde/ retrograde trafficking between the intracellular compartments. A C-terminal acidic cluster/ dileucine-based sorting motif is required for the sorting of lysosomal enzymes in the TGN.[44] The dileucine-based motif (Leu64, Leu65) is also important for sorting in endosomes.[45] The cytoplasmic domain of the CD-MPR contains three independent internalization signals, a pair of phenylalanine residues (Phe13, Phe18), a classical tyrosine-based sorting motif, and the dileucine-based motif.[46,47] The retrieval of the CD-MPR from late endosomes is mediated by a diaromatic sorting motif (Phe18, Trp19) and the palmitoylation of Cys34.[48,49] The phosphorylation of Ser57, which is part of a casein kinase-2 recognition sequence,[50] is believed to be a signal for cell surface delivery, probably by controlling the sorting of the receptor from endosomes to the plasma membrane.[51] In polarized cells, the CD-MPR is sorted to the basolateral plasma membrane. The basolateral sorting signal is located within the 19 juxta-membrane residues and depends on Glu11 and Ala17.[52,53]

The sorting signals in the cytoplasmic domains of MPRs are recognized by cytosolic adaptor proteins (AP) in distinct subcellular compartments mediating the package of the receptors in transport vesicles (see Chapter 3).[22] Additionally, the phosphorylation of serine residues located in the acidic cluster region of both MPR promote the recruitment of AP-1 to Golgi

membranes.[50,54] The anterograde transport of the MPRs is mediated by AP-1 and the Golgi-localized, γ-ear containing, ARF-binding proteins (GGAs, Fig. 1, step 4, whereas the retrograde transport from early and late endosomes requires AP-1/ phosphofurin acidic cluster sorting protein (PACS-1, Fig. 1, step 7) and TIP47/ Rab 9 (Fig. 1, step 8), respectively.[22] The internalization of MPR from the plasma membrane depends on AP-2 (Fig. 1, step 10).

Sorting Functions of Mannose 6-Phosphate Receptors

Both MPRs are required to guarantee the targeting of all newly synthesized M6P-containing proteins to lysosomes and they are not functionally redundant.[55] Reexpression studies either with CD-MPR or CI-MPR in MPR-deficient fibroblasts have demonstrated that both MPRs exhibit complementary binding properties. Furthermore, each MPR transports distinct subpopulations of lysosomal enzymes which is presumably due to the heterogeneity of the M6P recognition marker.[56,57] Thus, the CD-MPR exhibits a higher affinity for lysosomal enzymes containing one phosphomonoester, whereas the CI-MPR preferentially binds ligands with two phosphomonoesters.[58]

A variable portion of newly synthesized lysosomal enzymes escape the binding to MPR in the Golgi and are secreted (Fig. 1, step 12). These M6P-containing enzymes can be partially internalized and transported to the lysosomes through CI-MPR-mediated endocytosis. About 3-10% of total cellular MPR is localized at the plasma membrane at steady state and exchange with MPR cycling in the biosynthetic pathway (Fig. 1; steps 9, 11).

Delivery to the Endosomal/Lysosomal Compartment

Golgi or plasma membrane-derived transport vesicles containing MPR-ligand complexes fuse with the endosomal/prelysosomal compartment. Endosomes are characterized by an acidic intralumenal pH which is regulated and maintained by a balance between the rates of intralumenal proton pumping, counterion conductance and proton leak. Electrogenic V-ATPase pumps protons into endosomes which is regulated by passive anion flow through chloride channels.[59] Endosomal chloride channels CLC3, CLC5 and the lysosomal chloride channel CLC7 have been reported to be important for endosomal and lysosomal acidification, respectively.[60]

The low pH induces dissociation of lysosomal enzymes from MPR followed by delivery of the hydrolases to lysosomes and the recycling of MPR to the Golgi or plasma membrane. In the acidic milieu of endosomes, lysosomes or lysosome-related organelles, several precursor forms of lysosomal hydrolases are processed by autocatalytic proteolysis or other proteases. Thus, the low pH-induced cleavage of the inhibitory propeptide of cathepsin D leads to activation of cathepsin D.[61] In contrast, there are other examples, such as γ-interferon-inducible lysosomal thiol reductase[62] or aspartylglucosaminidase, where the N- or C-terminal processing of lysosomal hydrolases does not alter the activity of the protein.[63] pH-dependent oligomerization has been shown to be important for the stability of specific lysosomal enzymes such as arylsulfatase A.[64]

Lysosomal enzymes can be missorted and relocated under various pathological conditions, such as cancer,[65-67] Alzheimer's disease[68,69] or during apoptosis induced by oxidative stress.[70] It has been proposed that reduced expression level of the CI-MPR, increased expression of CD-MPR, or lysosomal membrane destabilization, respectively, are part of the underlying mechanisms.

Mannose 6-Phosphate Receptor-Independent Pathways

Studies of patients with I-cell disease (ICD), of mice lacking both MPRs, and several cell lines and primary cultured cells, have provided evidence for alternative, MPR-independent transport of newly synthesized lysosomal enzymes to lysosomes. Although all cells and tissues of ICD patients are deficient in phosphotransferase activity and lysosomal enzymes lack the M6P recognition marker, in many cell types and organs including liver, spleen, kidney and

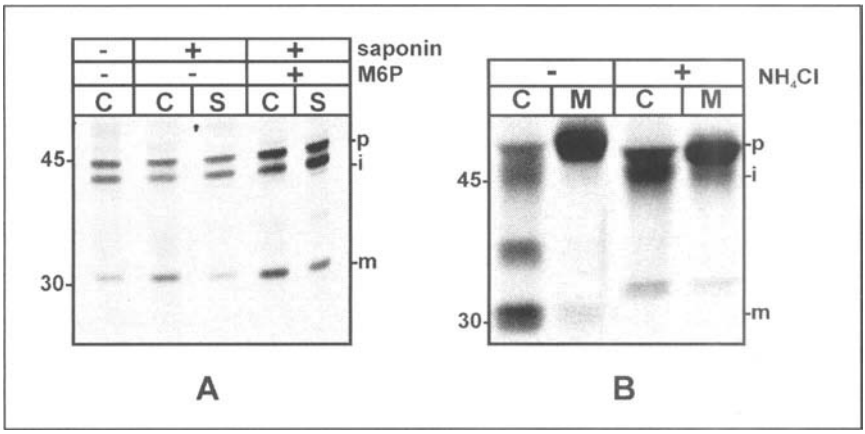

Figure 2. MPR-independent transport of cathepsin D. A) Human macrophages cultured for 20 days were labeled with [^{35}S] methionine for 30 min followed by a chase for 90 min. Thereafter the cells were either harvested or incubated with 0.1 % saponin in PBS in the presence (+) or absence (-) of 10 mM M6P at 4°C. After 30 min the saponin-containing buffer was removed (S), and the cells were washed with PBS and solubilized. Cell extracts and supernatant were used for immunoprecipitation. Note that half of the newly synthesized cathepsin D remained membrane-associated even in the presence of M6P. p, precursor; i, intermediate; m, mature form of Cathepsin D. B) Human macrophages were pulse-chase labeled in the presence (+) and absence (-) of 10 mM ammonium chloride (NH_4Cl). Cell extracts and media were used for immunoprecipitation of cathepsin D. The amount of cathepsin D in the medium was not affected by the presence of NH_4Cl indicating M6P-independent sorting (S.Tiede, N. Muschol, T. Braulke, unpublished results).

brain, normal lysosomal enzyme levels were found.[12,71] These studies were confirmed by experimental data from mice lacking both MPRs that resemble ICD patients in the clinical and biochemical phenotype.[72,73] Additionally, several reports have described MPR-independent lysosomal targeting of procathepsins in human hepatoma HepG2 cells,[74] mouse fibroblasts[75] or primary human macrophages (Fig. 2).[76] Furthermore, recent data on prosaposin have provided new insight in the mechanism of MPR-independent trafficking. Prosaposin exist in two isomeric forms of 65 and 70 kDa which are either targeted to the lysosome or secreted, respectively. In the lysosome, prosaposin is proteolytically cleaved into saposin A, B, C, and D which function as activator proteins for the hydrolysis of glycosphingolipids by specific lysosomal hydrolases.[77] Although prosaposin contains M6P residues, its direct intracellular targeting occurs in an MPR-independent manner involving sphingomyelin microdomains (lipid rafts) and the Golgi membrane protein sortilin.[78,79] In addition, the secreted 70 kDa prosaposin can be reinternalized and delivered to the lysosome in an MPR-independent manner mediated by the low density lipoprotein-receptor-related protein.[80] Finally, it has been reported that the MPR-independent transport of lysozyme in U937 cells is mediated by interaction with chondroitin sulphate.[81]

Perspectives

The complexity of enzymes in lysosomes seems to be enormous but reports describing newly discovered M6P-containing enzymes suggest that the list is by no means complete as shown by the growing number of cathepsins.[82] Lysosomal proteome analysis using the M6P modification as an affinity marker have proved to be a powerful tool to identify new proteins or the molecular basis for lysosomal disorders of unknown etiology.[83-85] It is likely that the newly discovered enzymes will have more restricted tissue distribution and have more unique

functions than those already fully characterized. The availability of naturally occurring animal models as well as the generation of gene targeted mouse models for several lysosomal proteins of known and unknown function contributes to a better understanding of the pathogenesis of storage diseases and to dissolve their role in lysosome metabolism.[86,87] Furthermore, gene array analysis in tissues of the animal models will provide valuable clues understanding regulatory and compensatory mechanisms in lysosome biology. Finally, the determination of the X-ray structure of wild type and mutant lysosomal enzymes is required to elucidate the formation, stability and function of multimeric complexes in the lysosome on the molecular level[64] and will open up possibilities of designing structure-based drugs aimed to restore the activities of defective lysosomal hydrolases.

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CHAPTER 3

Adaptor Proteins in Lysosomal Biogenesis

Peter Schu*

Abstract

Lysosomal membrane proteins and soluble lysosomal proteins are transported from the trans-Golgi network (TGN) to endosomes and lysosomes via coated-vesicles which bud from the donor compartment and are transported to and fuse with the proper acceptor compartment. The proteins forming the vesicle-coat bind to the cytoplasmic domains of the cargo proteins and recruit additional proteins like clathrin to the site of vesicle formation. These proteins are hence called adaptor proteins or adaptor-protein complexes and their subunits are called adaptins. The family of heterotetrameric adaptor-protein complexes consists of AP-1, AP-2, AP-3 and AP-4 and all four are required for lysosome biogenesis. They are ubiquitously expressed in mammals and many of the adaptins also exist as tissue-specific isoforms encoded by different genes or generated by alternative splicing. Adaptor-protein complexes are compartment specific proteins and recruit their specific accessory proteins to the site of vesicle formation, which is believed to regulate vesicle budding and fission and vesicle transport.

Heterotetrameric Adaptor-Protein Complexes

A family of four heterotetrameric adaptor-protein complexes (Table 1 and Fig. 1), AP-1, AP-2, AP-3 and AP-4 exists. AP-1, AP-3 and AP-4 have been localized to the TGN and endosomes, whereas AP-2 is exclusively localized to the plasma membrane, where it mediates endocytosis. All four complexes have the same subunit composition (Fig. 1).[1] They consist of two large subunits of > 110 kDa. Both are subdivided in an N-terminal globular domain and a the C-terminal globular domain separated by a long proline-rich flexible stalk. The C-terminal part is therefore referred to as the 'ear' domain (Fig. 1). One subunit of these pairs shows limited sequence homology among the AP-complexes and is named γ1 in AP-1, α in AP-2, δ in AP-3 and ε in AP-4. The second large subunits of the complexes show the highest sequence conservation among the AP-subunits and were named β1- to β4-adaptin. The AP-complexes further contain one medium sized μ-adaptin of ~50 kDa (μ1 to μ4) and one small σ-adaptin subunit of ~20 kDa (σ1 to σ4) (Table 1). The crystal structures of AP-2 and AP-1 have recently been solved.[2,3] Despite the differences in the sequences of the α- and γ1-adaptins both form homologous ternary structures. The complexes can be subdivided in two hemi-complexes formed by γ1/σ1 or α/σ2 and by the μ/β adaptins, which bind to each other by interactions of the two large subunits. In the heterotetrameric complex all subunits have surface contacts with each other. μ1-adaptin deficient cells have reduced expression levels of σ1-adaptin.[4] This indicates that there are cooperative interactions between the subunits, which are important for complex formation and function.

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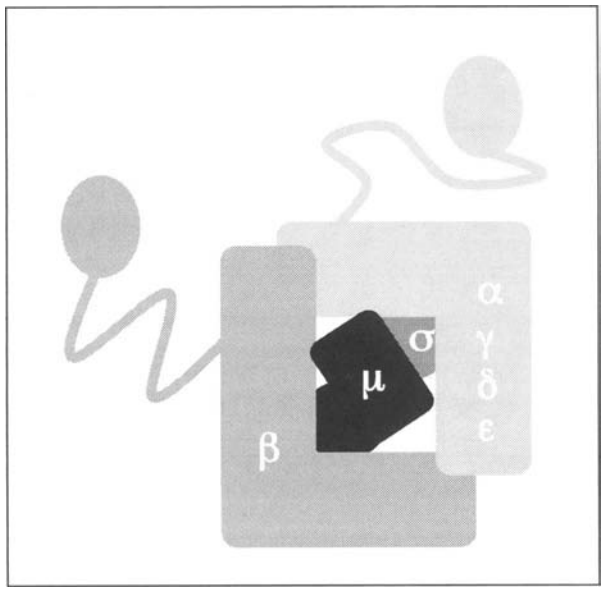

Figure 1. Heterotetrameric adaptor protein complex subunit composition and subunit interactions as shown by the crystal structures of the AP-1 and AP-2 complexes. Due to the high primary and secondary structure conservation it is expected that AP-3 and AP-4 complexes are formed by analogous adaptin-interactions. Both large subunits bend and interact with each other via their N-termini and the C-terminal domains of their N-terminal globular domains. Both 'ear'-domains are connected by a long proline rich unstructured domain allowing them to reach out into the cytoplasm. σ-adaptins bind to the bend of γ1- or α2-adaptins. The N-terminal domains of the μ-adaptins have a ternary structure comparable to the σ-adaptins and anchor the adaptins to the bend formed by the β-adaptins. The C-terminal, sorting motif binding domain of the μ-adaptins is localized to a groove formed by the two large adaptins. This domain might flip out of the core structure to allow binding of sorting motifs to μ-adaptins (see text for details).

AP-1 and AP-2 are the classical clathrin-coated vesicle adaptor-protein complexes.[5] Clathrin assembles into a hexgonal lattice on membranes, which during the process of vesicle formation, changes into a basket made out of pentagons on vesicles. The basic assembly unit is the clathrin triskelion, which is composed by three clathrin heavy chains and three clathrin light chains.[6] The clathrin heavy chains trimerize with the C-termini in the center to form the triskelion. Each 'leg' of the triskelion can be divided in a proximal segment, a distal segment and an N-terminal globular domain, which is separated by a flexibel domain. This linker allows the N-terminal domain to project inwards. Three clathrin light chains bind to the heavy chains at their proximal segment. These triskelions assemble into hexagons and pentagons.[5,7] Clathrin self-assembly is regulated by the light chains.[8] The N-terminus of the clathrin heavy chain has a propeller configuration and binds to the consensus motif LLD/NLD in the C-terminal region of β-adaptins of AP-1 and AP-2 and other coat-proteins.[6] Although the β3-adaptin of AP-3 binds clathrin in-vitro, in-vivo studies indicate that AP-3 function is clathrin-independent.[9] The vesicle coat is disassembled to allow recycling of the coat proteins and to enable membrane fusion. Disassembly of the clathrin coat involves the HSC70 (heat-shock-cognate 70) ATPase and the cochaperone auxilin, which in vitro bind to the clathrin light chains.[10-14]

The AP-1 complex exists as the ubiquitous AP-1A and the polarized epithelia specific AP-1B complex. The AP-1B complex contains the μ1B-adaptin, which is 87% homologous to the ubiquitously μ1A-adaptin.[15] Other epithelia specific AP-1 adaptin isoforms are not known. AP-1A and AP-1B are expressed in polarized epithelia and are localized to different subdomains of the TGN. The

Table 1. Family of heterotetrameric adaptor-protein complexes

Complex	Large Variable	Medium	Small	Large
	≥110 kDa	~50 kDa	~20 kDa	≥110 kDa
AP-1A	γ1	μ1A	σ1A/s1B/s1C	β1
AP-1B		μ1B		
vAP-1	γ2	?	σ1B	?
AP-2	α_A (large) α_A (small) α_C	μ2	σ2	β2
AP-3A	δ	μ3A	σ3A	β3A
AP-3B		μ3B	σ3B	β3B
AP-4	ε	μ4	σ4	β4

Blanks indicate that the common adaptin is incorporated into the complex. Question-marks indicate unknown subunit composition.

AP-1B complex mediates basolateral sorting of the low-density-lipoprotein-receptor in polarized epithelia.[16-18] σ1 adaptin is present in three ubiquitously expressed isoforms, σ1A, σ1B and σ1C, which are over 85% homologous. Both σ1A and σ1B interact in-vitro with γ1-adaptin.[19,20] σ-adaptins may have a structural function in complex assembly and stability (Fig. 1). A ubiquitously expressed γ2 isoform exists, which is also localized to the TGN, but it does not colocalize with clathrin in vivo.[19,20] It binds to σ1B, but other adaptins of a γ2 adaptor-protein complex have not been characterized.

The AP-3 complex is present as the ubiquitously expressed AP-3A and the neuron specific AP-3B complex. Both share the δ-adaptin, but AP-3B contains the β3B, μ3B and σ3B adaptins.[21-23]

The AP-4 complex is present in only one ubiquitously expressed form and is only found in higher eukaryotes.[24,25]

The AP-2 complex consists in two isoforms containing different α-adaptins, a 108 kDa α_A-adaptin and a 104 kDa α_C-adaptin. Both isoforms are encoded by different genes and are ubiquitously expressed. Their relative expression levels however vary, indicating that they fullfill different functions, although both isoforms are found on the same vesicles. α_A-adaptin mRNA is alternatively spliced in the brain, introducing an additional sequence into the proline-rich flexible stack, which links the N- and C-terminal globular domains.[26]

Membrane Binding of Adaptor-Proteins

The γ1-, α-, δ- and ε-adaptins of the complexes show the least sequence homologies among all adaptins and are thus the most individual subunits of the complexes. Therefore they might be responsible for the compartment specific binding of the adaptor complexes. Furthermore γ1- and δ-adaptin have been shown to mediate complex formation.[27,28] γ1-Adaptin and α-adaptin contribute to the membrane binding of the complexes via there specificities for the membrane lipids phosphatidylinositol-4-phosphate (PI-4-P) and phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2) respectively.[2,3,29-31] PI-4-P is enriched in the TGN and is found on early endosomes, which are characterized by a high PI-3-P content.[32] PI-4,5-P_2 is enriched in the plasma membrane. The C-terminal 'ear'-domains of both recruit a specific subset of so called accessory proteins to the site of vesicle formation, which are involved in the vesicle budding and fission processes.[33-40] β-1, -2 and -3 adaptins have a high affintity clathrin-binding site, however only AP-1 and AP-2 require clathrin for function, but not AP-3.[9] β1-adaptin binds the microtubulie based plus-end directed KIF13A motor protein.[41]

Membrane binding of AP-1, AP-3 and AP-4 also requires the small GTPase Arf-1 in its GTP-bound form for high affinity membrane binding, whereas membrane binding of AP-2 does not require the presence of Arf-1 or of a homologue at the plasma membrane. Arf-1 has been crosslinked to both large adaptins of AP-1 and AP-3 and it was shown to bind to ε-adaptin of AP-4.[42,43] Membrane binding of γ2-adaptin is Arf-1 independent as the binding is not blocked by the inhibition of Arf-1 GDP-exchange proteins of the Sec7-domain family.[19,20] Membrane binding of the adaptor-complexes also requires in the target membrane the presence of a cytoplasmic peptide sequence containing a sorting motif for binding to the respective adaptor-protein. Sorting motifs bind to the μ-adaptins of the complexes, to β-adaptins and to γ1/σ1 and α/σ2 hemi-complexes.[44-46] Binding of the common tyrosine-based YxxQ-sorting motifs, where Q is a large hydrophobic residue, is best characterized by cocrystallization of the C-terminal domain of μ2-adaptin with peptides corresponding to the sorting motifs of TGN46 and of the epidermal growth factor receptor. μ2-residues mediating YxxQ-binding are conserved in all μ-adaptins.[47,48] Residues surrounding the YxxQ-motif determine the affinities for binding to the μ-adaptins. The second large family of sorting motifs are di-leucine based motifs, often preceeded by a cluster of acidic residues. Such motifs appear to be able to bind to β-adaptins as well as to γ1/σ1 and α/σ2 hemi-complexes.[44,46] Membrane binding of adaptor-proteins is controlled by adaptin-phosphorylation and -dephosphorylation. Cytoplasmic adaptor-protein complexes contain phosphorylated β-adaptins. Upon membrane binding β-adaptins become dephosphorylated, while μ-adaptins become phosphorylated. μ-adaptin phosphorylation appears to induce flipping of the μ-adaptin C-terminal domain out of the adaptor-core complex.[2,3,49] This appears to be essentiel for YxxQ sorting motif binding, because the residues mediating the binding are hidden in the compact conformation by β-μ adaptin interactions. A trimeric γ1-σ1-β1 is not able to bind to membranes in vivo or in vitro.[4] The respective kinases and phosphatases for AP-1 have not been identified, but more is known about enzymes for AP-2 and clathrin phosphorylation cycles.[13,50-52]

Adaptor-Protein Sorting Pathways

The AP-2 complex is able to mediate endocytosis of lysosomal membrane proteins and of the mannose-6-phosphate receptors MPR46 and MPR300, which mediate the sorting of soluble lysosomal enzymes between the TGN and endosomes. The MPR300 only is able to endocytose secreted lysosomal enzyme like cathepsin D, which in some tissues might be a special form of lysosomal enzyme sorting.[53] The MPR300 function in endocytosis is essentiel for the lysosomal degradation of excess insulin-like growth factor II (see also Chapter 2). Only the lysosomal acid phosphatase (LAP), CD1b and endolyn are sorted to lysosomes via the plasma membrane and their sorting depends on AP-2 function.[54-57] They are directly secreted from the TGN to the plasma membrane and do not bind to the TGN adaptor-protein complex AP-1. They do however bind to AP-2 and are endocytosed to early endosomes from which they reach late endosomes and lysosomes (Fig. 2).

The AP-1 complex is predominantly found on the TGN, but has also been detected on endosomes. It is, with the monomeric GGA adaptins, required for TGN to early endosome transport of both MPRs.[58] This is indicated by the detection of the Golgi-precursor form of their cargo cathepsin D in early endosomes and by the accumulation of the MPRs in early endosomes of AP-1-deficient cells, derived from a μ1A-adaptin 'knock-out' mouse. These cells are unable to transport the MPRs back to the TGN, so they can not bind enzymes in the biosynthetic pathway (Fig. 2).[4,59] Due to the MPR mislocalization to early endosomes the Golgi-precursor form of cathepsin D is initially secreted by these cells, but is then bound by MPR300 at the plasma membrane and endocytosed by the AP-2 and transported to early endosomes. Cathepsin D is then transported to lysosomes.[4,59] MPRs are directly transported back to the TGN from early endosomes, but it is not known, whether this involves AP-1 mediated transport vesicle formation on early endosomes or whether AP-1 is transporting

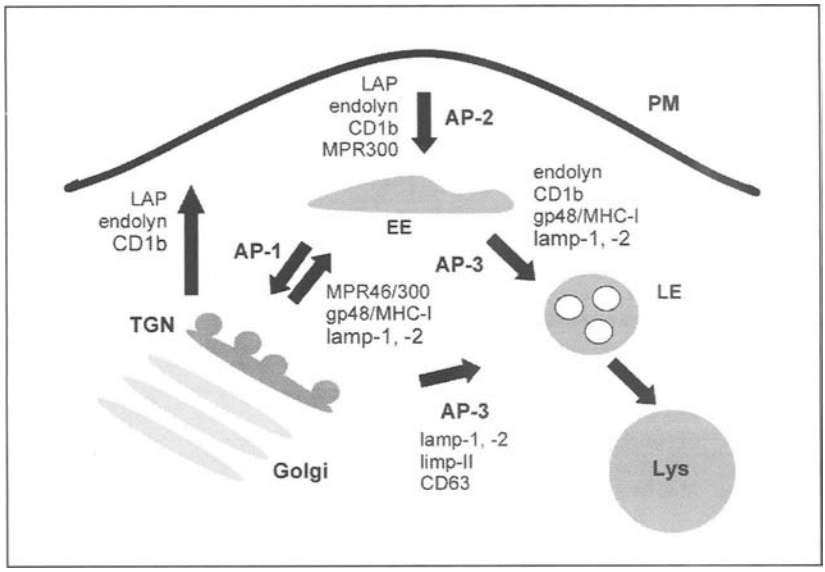

Figure 2. Sorting pathways in lysosomal biogenesis mediated by the adaptor proteins AP-1, AP-2 and AP-3. TGN: trans-Golgi network; PM: plasma membrane; EE: early endosome; LE: late endosome; Lys: Lysosome.

essentiel factors for the retrograde pathway from the TGN to endosomes.[60,61] MPRs have also been detected in late, multivesicular endosomes by immunogold electron microscopy and thus a fraction of MPRs reaches the TGN via late endosomes. AP-1 mediated TGN to early endosome sorting is essential for intracellular lysosomal transport of a gp48/MHC-I complex.[62] gp48 is a cytomegalovirus protein, which binds to MHC-I in the endoplasmic reticulum and directs MHC-I transport to lysosomes, thus preventing its appearance at the plasma membrane.[63] This is one mechanism the virus uses to escape the immune system. After AP-1 mediated TGN to endosome transport the gp48/MHC-I complex is transported to lysosomes by the AP-3 complex, because its degradation is delayed in AP-3-deficient cells, but it is not missorted to the plasma membrane as in AP-1-deficient cells. Thus AP-1 and AP-3 act consecutively on the same TGN to lysosome transport pathway.[62] These functional data are supported by semi-quantitative immunogold electron microscopy studies, which localize most of AP-3 to endosomes and only a minor fraction to the TGN.[64]

Besides LAP, endolyn and CD1b, lysosomal membrane proteins like Lamp1, -2, Limp-II and CD63 are sorted intracellularly and only a minor fraction of these proteins can be detected at the plasma membrane.[55,65,66] The AP-3 is required for intracellular lysosomal transport of the lysosome-associated-membrane protein 1 (Lamp1). In AP-3-deficient cells Lamp1 lysosomal transport is delayed, because it recycles between endosomes and the plasma membrane before reaching lysosomes.[67,68] It is not known, whether TGN sorting of Lamp1 is mediated by AP-3 or AP-1. In AP-1-deficient cells no missorting of Lamp1 is detected demonstrating that AP-1 is not essentiel.[4] Therefore AP-3 might direct Lamp1 from the TGN directly to late endosomes as it has been described for some cell types.[69] The luminal domain of Lamp1 influences its sorting by unknown mechanisms, which makes it difficult to clearly demonstrate a function of the cytoplasmic domain and thus for adaptor-proteins in lysosomal transport.[70] It has been demonstrated that a chimera carrying the cytoplasmic and transmembrane domains of Lamp1 and luminally the ER-targeting sequence of cathepsin D, two secretogranin I tyrosine-sulfation domains and avidin, is transported from the TGN first to

early endosomes, possibly by AP-1, before reaching late endosomes and lysosomes.[71] Thus there might be a redundancy in AP-1 and AP-3 sorting at the TGN. In the absence of AP-3, AP-1 might be able to sort some proteins, like Lamp1 or Lamp2, into a lysosomal pathway bypassing the plasma membrane (Fig. 2). This interpretation is supported by the phenoytpes of AP-1 and AP-3 mouse 'knock-out' models. While AP-1-deficient mice die in utero, AP-3-deficient animals are viable and fertile and show only defects in a subset of cell types.[4,27,28] They show neurological defects due to AP-3B function in vesicle budding from endosomes in the synapse and due to sorting of zinc and GABA transporters to neurosecretory vesicles.[27,72] The mice have defects in the biogenesis of some specialized lysosomes, like melanosomes and dense-granula in platelets, causing pigmentation defects and delayed blood coagulation. These mice provide a model for the Hermansky-Pudlack syndrome described in humans. Patients have also defects in the biosynthesis of specialized lysosomes and show pigmentation defects, impaired blood coagulation and ceroid deposition.[27,68,73] The pigmentation defect is caused by impaired sorting of tyrosinase to melanosomes, the key enzyme in melanin biosynthesis.[74-77]

Also the AP-4 has been localized to the TGN and endosomes, but its function is not known.[24,25] It might be involved in endosome and lysosome biogenesis in polarized cell types by targeting proteins from endosomes below the apical plasma membrane to basolateral endosomes.[78,79]

Coadaptor Proteins

The AP-2 complex forms a mixed coat with β-arrestin, which binds to the cytoplasmic domain of activated β-adrenergic receptors and to clathrin.[80] The monomeric AP-180 (180 kDa) and its homologue CALM (clathrin assembly myeloid leukemia protein) form clathrin-coated vesicles in-vitro and display a higher affinity towards clathrin than AP-1 or AP-2.[81] AP-180 is expressed ubiquitously and exists in a neuronal isoform. AP-180 also binds to AP-2 so they form mixed coats.[33,82] Inhibiton of AP-2 function did not completely abolish clathrin-mediated endocytosis indicating that these monomeric adaptor-proteins can also function independent of AP-2.[83-85]

Three homologous monomeric adaptor-proteins have been identified based on their sequence homology to the C-terminal 'ear' domain of γ1-adaptin. They were named GGA1 to 3 for Golgi-localised, γ-adaptin homologue and ARF-binding protein.[86-88] The γ-adaptin homologous C-terminal domain is called the 'appendage' domain. The N-terminal VHS domain binds to cargo proteins. This domain is followed by the GAT domain, which binds Arf1. GAT and appendage domains are connected by a long flexible hinge domain, which binds to the clathrin-heavy-chain, its own VHS domain and the γ-adaptin 'ear'-domain.[89] All GGAs have been localised to the TGN, but can also be detected on endosomes. On endosomes they bind to ubiquitin-tagged cell surface receptor proteins and appear to direct them into the late endosomal multi-vesicular-body pathway for subsequent degradation in lysosomes.[90,91] GGAs are recruited to the TGN by directly interacting with Arf1.[92] The VHS domain binds to MPR46 and MPR300 and it has been proposed that GGAs recruit MPRs and clathrin to the site of vesicle formation and then pass these proteins on to the AP-1 complex.[89] The GGA proteins differ in their affinities to the sorting motifs in MPRs, but the in-vivo significance of this is not known. GGAs appear to function in concert, because 'knocking-down' one isoform by RNAi causes proteolysis of the remaining isoforms. Thus it is also possible that these monomeric adaptor-proteins function as adaptor-protein complexes.[93] It can not be excluded that GGAs function independent of AP-1, but they could not be detected on purified clathrin-coated vesicles.[34] However accessory proteins bind specifically to the GGA appendage domain and the γ1-adaptin 'ear'-domain indicating that AP-1 and GGA proteins can function independently in protein sorting.[37]

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CHAPTER 4

Lysosomal Membrane Proteins

Paul Saftig*

Abstract

The lysosomal limiting membrane has multiple functions including acidification of the lysosomal matrix, sequestration of lysosomal enzymes, mediation of fusion events beween lysosomes and other organelles, and transport of degradation products to the cytoplasm. Lysosomal membrane proteins are usually highly glycosylated proteins decorating the luminal surface of lysosomal membranes. LAMP-1, LAMP-2 and LIMP-2 are the most abundant components of this membrane. Experiments on knockout mice have demonstrated that these proteins are important for normal cell physiology and that they can be involved in pathological conditions. The deficiency of LAMP-1 causes only a mild phenotype and also no apparent lysosomal dysfunctions. It is likely that the lack of LAMP-1 may be compensated by the structurally related LAMP-2. A role for LAMP-2 in the so-called chaperone-mediated autophagy has been described. Furthermore, LAMP-2 deficiency in mice has revealed roles in lysosomal enzyme targeting, autophagy and lysosomal biogenesis. LAMP-2-deficient mice exhibit a similar phenotype to individuals with Danon disease, which is caused by mutations in the LAMP-2 gene. Experiments on LIMP-2 knockout mice and overexpression studies also suggested specific functions for this protein in maintaining normal lysosomal biogenesis. Apart from the major proteins of the lysosomal membrane, about 20 less abundant or transient components of this membrane have been described. Although it is known that mutations in some of these proteins are associated with human disease, for most of the lysosomal membrane components the actual function is yet to be determined.

Lysosomes represent the major intracellular site for the degradation of a variety of macromolecules, including proteins, carbohydrates, nucleic acids, and lipids, which are internalized from the extracellular space by endocytosis, delivered by fusion with phagosomes or autophagosomes, or derived from the biosynthetic pathway. Lysosomes are enriched with acid hydrolases. One crucial role of the membrane enclosing late endosomes and lysosomes is to separate the potent luminal acid hydrolases from other cellular constituents and so protect them from unwanted degradation.

Cholesterol, dolichol derivatives, bismonoacylglycerol and phospholipids, including sphingomyelin are the major lipids of the 7-10 nm-thick lipid-bilayer of the lysosomal membrane.[1] Although the lysosomal membrane is usually impermeable to macromolecules and intermediate digestion products, it allows small molecules of up to 200 Da to diffuse freely. Saturable efflux or influx kinetics of radiolabelled metabolites into lysosomes were used to demonstrate the existence of more than 20 lysosomal membrane transporters. To date, however, only three of these transporters, cystinosin and sialin, involved in the transport of cystine and sialic acid, have been identified. Recently a third transporter, LYAAT-1, has been molecularly and functionally characterised. This transporter is possibly involved in the lysosomal export of neutral amino acids.[2] Some lysosomal storage diseases with intralysosomal storage of products of

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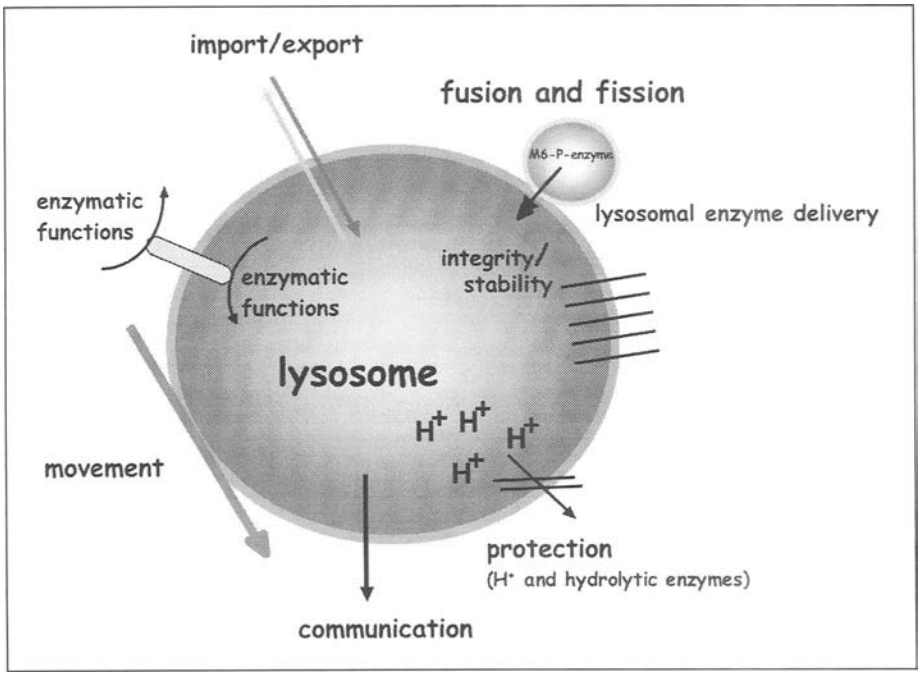

Figure 1. Proposed functions of lysosomal membrane proteins. Due to their central role at the interface between the lysosomal lumen and cytosol, it has been postulated that major but also less abundant lysosomal membrane proteins are involved in lysosomal import and export processes, in interaction with microtubules (movement), in protection of the lysosomal membrane against degradation, in maintenance of lysosomal integrity and stability, in enzymatic functions, and in fusion and fission events.

lysosomal hydrolysis have also been described, which gives further evidence for the existence of lysosomal transporters.[3]

The unique constitution of the lysosomal membrane is highlighted by an extremely rich content in carbohydrates. However, it was already discovered that certain protein components of the lysosomal membrane mediate a number of the essential functions of this organelle. For example, the acidification of the lysosomal lumen by a proton transport, as well as translocation of amino acids, fatty acids and carbohydrates resulting from hydrolytic degradation, and of nutrients liberated by specific lysosomal hydrolases (vitamin B12 and cholesterol) has been attributed to integral proteins of the lysosomal membrane (Fig. 1). Lysosomal membrane proteins are also thought to be involved in the interaction and fusion of the lysosomes with themselves, as well as with other membrane organelles, including endosomes, phagosomes and the plasma membrane.[4]

In the early eighties two groups analysed proteins of the lysosomal membrane. They identified two abundant high molecular weight glycoproteins[5,6] which were unique to lysosomes and not present in the plasma membrane. A more thorough understanding of the protein composition was achieved after the production of monoclonal or polyclonal antibodies directed against enriched lysosomal membranes.[7,8] In these studies, highly glycosylated integral proteins in the 90-120 and 30-85 kDa range were identified and shown to be enriched in late endosomes and/or lysosomes (Fig. 2). These proteins were designated lysosome-associated membrane proteins (LAMPs), lysosomal membrane glycoproteins (LGPs) or lysosomal integral membrane proteins (LIMPs). Chen and coworkers first described two different major glycoproteins, termed LAMP-1 and LAMP-2 (Chen et al 1985). A few years later, Lipincott-Schwartz and Fambrough

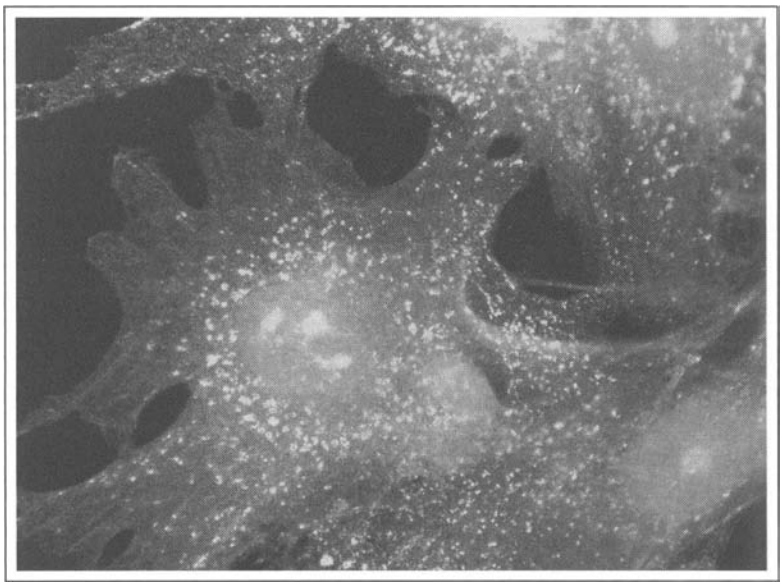

Figure 2. Immunofluorescence image of a mouse embyronic fibroblast stained for the major lysosomal membrane protein LAMP-2. Note the ring-like vesicular lysosomal structures localised in the perinuclear region. Ring-like staining indicates that the vast majority of label is localised to the limiting membrane of the endo/lysosomal vesicles.

isolated chicken LAMP-1 (LEP-100).[9] Other groups isolated both proteins as major carriers for poly-N-acetyllactosamines[10,11,12] containing complex carbohydrates with various ligand and antigenic properties.

LAMPs and LIMPs as Major Components of the Lysosomal Membrane

It has been estimated that LAMPs and LIMPs (Fig. 3) are tightly packed and represent more than 50 % of the total membrane protein of late endosomes and lysosomes. In P388 macrophages, LAMP-1 comprises about 0.1% of total cell protein, corresponding to about 2 x 10^6 LAMP-1 molecules/cell.[13] The collective abundance of both LAMP-1 and LAMP-2 has been estimated to be high enough to form a nearly continuous carbohydrate coat on the inner surface of the lysosomal membrane.[14]

More extensive studies have been carried out on LAMP molecules than on other lysosomal membrane proteins. LAMP-1 and LAMP-2 cDNAs from different species have been isolated and studied. Both are type-I membrane proteins, with a large luminal ectodomain divided into two homologous domains by a hinge region rich in proline, serine and threonine, a transmembrane region and a short cytoplasmic tail. The intraluminal domain is glycosylated with some O-glycans and a large number (16-20) of N-glycans, most of which are of the complex poly-N-acetyllactosamine type. The conserved cytosolic tail of LAMP-1 and LAMP-2 possesses 11 residues and contains the necessary information for their intracellular targeting after biosynthesis (see below). Despite their 37% amino acid sequence homology, LAMP-1 and LAMP-2 are distinct proteins which probably diverged relatively early in evolution as evidenced by their localisation on different chromosomes.[4]

The protective function against degradation by lysosomal hydrolases was assigned to the heavy glycosylation of both LAMP proteins.[4,15] The inner surface of the lysosomal limiting membrane is lined by a layer of carbohydrates generating a continuous glycocalyx.[16] Depletion

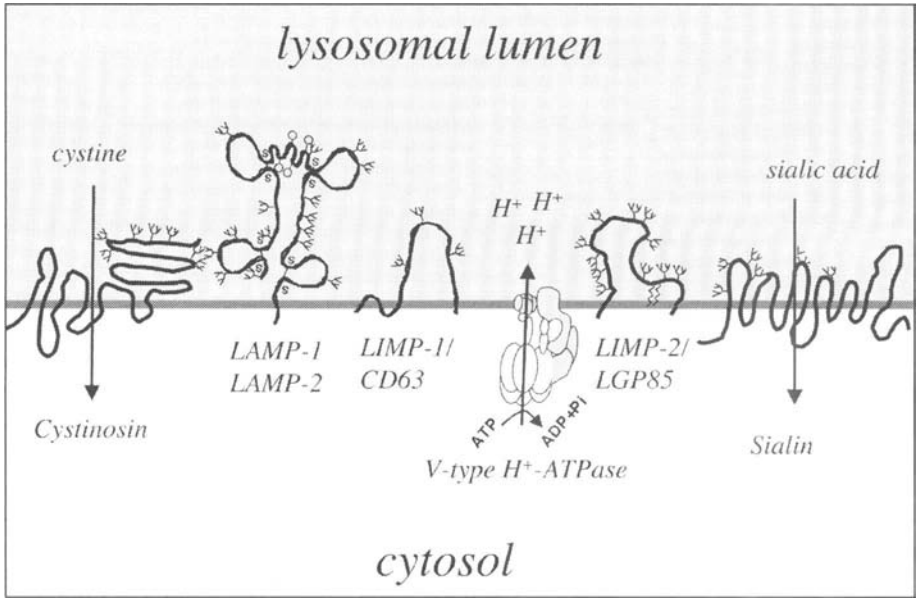

Figure 3. Schematic drawing of the topology of the major lysosomal membrane proteins. Putative N-glycosylation (antennae) and O-glycosylation (grey circles) sites are indicated.

of the Asn-linked oligosaccharides from lysosomal membrane proteins using endoglycosidase H feeding to cultured cells, resulted in the rapid degradation of LAMP-1 and LAMP-2.[17] However, LIMP-2/LGP85 was relatively stable in the same cells, indicating that Asn-linked oligosaccharides are not necessary for the stability of all lysosomal membrane proteins. Interestingly, the content of poly-N-acetyllactosamines in LAMP-1 correlates with the differentiation[10,18] and metastatic potential[19-21] of tumor cells.

One of the early functions for a lysosomal membrane protein was the description of LAMP-2 as a receptor for the selective uptake and degradation of proteins by lysosomes.[22] The substrate proteins of this uptake contain a pentapeptide motif related to KFERQ.[23] The pathway was shown to be activated in confluent lung fibroblast and CHO cell cultures by serum withdrawal, and in rat liver by prolonged starvation (reviewed in ref. 24). In addition to LAMP-2, the heat shock cognate protein of 70 kDa (Hsc73) is involved. Other chaperones may also be required for the transport, including Hsp90, Hsp40 and some others.[25] A detailed description of chaperone-mediated autophagy and the proposed role of LAMP-2 in this process is found in Chapter 15.

LAMP-Deficient Mice

Although much was known about the structure and lysosomal trafficking of LAMP-1 and LAMP-2 the proposals for their physiological functions were only of a hypothetical nature. In order to understand the possible contribution of one of the major components of the lysosomal membrane mice were generated that are deficient in LAMP-1 and/or LAMP-2. Despite its abundance in the lysosomal membrane, the deficiency of LAMP-1 is apparently well-tolerated. LAMP-1 deficient mice were viable and fertile. Apart from a mild regional astrogliosis and an altered immunoreactivity to cathepsin D in the brain, all tissues of these mice were normal.[26] Histological and ultrastructural analyses of all other tissues did not reveal abnormalities. The properties of lysosomes from LAMP-1 deficient cells, including enzyme activities, luminal pH, osmotic stability, density, morphology, subcellular distribution, and lysosomal enzyme processing, were

similar to those of control cells. An upregulation of LAMP-2 protein was observed in the kidney, spleen and heart of LAMP-1 deficient mice, while LIMP-2/LGP85 levels were unaffected. This upregulation was also evident in tissues lacking only one LAMP-1 allele, which suggests a coupling in the regulation between the LAMP-1 and LAMP-2 levels.

LAMP-2 deficient mice are more severely affected and about 50% die between 20 and 40 days of age.[27,28] Both the mice that died early and those that survived were smaller than the wild type animals. The surviving mice were fertile and had an almost normal life span. The major pathological feature was an extensive accumulation of autophagic vacuoles in several tissues, including liver, pancreas, spleen, kidney, skeletal muscle, heart, capillary endothelium, intestinal wall, lymph nodes and neutrophilic leukocytes. Both skeletal and cardiac muscle cells showed an accumulation of autophagic vacuoles. The LAMP-2 deficient mice had large hearts with decreased contractility and abnormal and degenerating myocytes. The physiological importance of LAMP-2 is supported by the finding that LAMP-2 deficiency is the primary defect in Danon disease,[29,30] a lysosomal glycogen storage disease with normal acid maltase activity.[31] Danon disease is characterised by fatal cardiomyopathy, variable mental retardation, and mild skeletal myopathy. Vacuoles in skeletal and cardiac muscle containing glycogen and cytoplasmic degradation products are the pathological hallmark of this disease.

The aberration in autophagy, due to a lack of LAMP-2, appeared to have a wider tissue distribution in mice than in human patients.[28] LAMP-2 deficiency in mice causes the accumulation of autophagic vacuoles in pancreatic, hepatocytic, endothelial cells and leucocytes. This accumulation was also preserved in hepatocytes isolated from the knockout mice. In addition, the degradation of long-lived proteins, which mainly occurs via autophagy in hepatocytes, was reduced compared to control cells.[27] Quantitative electron microscopy indicated that the half-life of autophagic vacuoles was prolonged,[32] suggesting that retarded consumption, rather than increased formation of autophagic vacuoles, was the cause of their accumulation. Fusion of the endocytic organelles and the delivery of the vacuolar proton pump was apparently normal. LAMP-2 deficient hepatocytes revealed elevated secretion of a subset of lysosomal enzymes, as well as impaired processing of cathepsin D. The steady-state level and half-life of the 46 kDa mannose-6 phosphate receptor were severely reduced, with a shift in its localisation from the TGN to autophagic vacuoles, suggesting that LAMP-2 deficiency either directly or indirectly leads to impaired recycling of 46 kDa MPR from endosomes to the TGN. This in turn, could explain the partial mistargeting of a subset of lysosomal enzymes. Autophagic vacuoles may therefore accumulate due to an impaired capacity for lysosomal degradation.

The embryonic lethal phenotype of mice deficient in both LAMP-1 and LAMP-2 supports the hypothesis that the structurally-related LAMP-1 might compensate in part for the loss of LAMP-2. Double deficient embryos die at E.15 and are characterised by an accumulation of autophagic vacuoles in the majority of embryonic tissues.[78] Cells derived from these embryos still have active lysosomes although with a different density and light microscopical apperance. Surprisingly, in cells lacking both LAMP-proteins the lysosomal lipid metabolism/trafficking is severely affected.[78]

LIMP-Deficient Mice

The type III membrane proteins LIMP-1 and LIMP-2/LGP85 are also major lysosomal membrane proteins. Whereas LIMP-2/LGP85 traverses the membrane twice,[33] LIMP-1 (CD63) spans the lysosomal lipid bilayer four times.[34] The signal peptide of both proteins is probably not cleaved off, and both proteins contain complex carbohydrate structures in their luminal domain.

LIMP-1/CD63 belongs to the so-called tetraspanins (Fig. 3). In addition to localisation in late endosomes and lysosomes, it is also present in the secretory granules of certain hematopoetic cells (ref. 35 and references therein). This molecule may play a role in cell activation and the fusion of secretory granules with the plasma membrane. This tetraspanin has numerous interaction partners, including other tetraspanins such as CD82; the MHC class II molecules

HLA-DR, HLA-DM, and HLA–DO; several integrins; and phosphatidylinositol 4-kinase (ref. 36 and references therein). The subcellular localisation of CD63 and its interaction with the adaptor complexes and phosphatidylinositol 4-kinase suggest that this tetraspanin may be involved in protein trafficking. Recent experiments suggest that LIMP-1/CD63 enhances the internalisation of the H,K-ATPase beta-subunit and may serve as an adaptor protein that links its interaction partners to the endocytic machinery of the cell.[36] Knockout mice for LIMP-1/CD63 were generated. These mice were viable and did not show an overt phenotype. However, we and others[37] found evidence that there are two LIMP-1/CD63 genes in mice. It may be possible that the actual consequence of the loss of LIMP-1/CD63 is masked by a residual expression of the second and nontargeted mouse gene (P. Saftig, unpublished results).

LIMP-2/LGP85 (Fig. 3) may also be involved in lysosome/endosome biogenesis. Overexpression of LIMP-2/LGP85, but not of LAMP-1 or LAMP-2, was shown to result in the accumulation of large swollen vacuoles that have both early and late endosomal and lysosomal characteristics.[38] On electron microscopy, the large vacuoles appear electron-lucent with only occasional luminal membranes, suggesting that the invagination of internal vesicles may be impaired. Pulse-chase experiments showed that the large vacuoles were not initially derived from lysosomes. Coexpression of dominant-negative Rab5b with LIMP-2/LGP85 totally inhibited the formation of the large swollen vacuoles, indicating that the Rab5 function was necessary for their appearance. All these results[38] suggest that LIMP-2/LGP85 may control the balance between vesicle invagination versus vesicle budding from the limiting membrane of endosomal compartments and, thus, that they play a role in the biogenesis and maintenance of endosomes and lysosomes. It is possible that overexpression of LIMP-2/LGP85 may cause a dispersal of the budding machinery, which might be due to an impaired recruitment of a cytoplasmic factor involved in vesicular fission and/or fusion.

LIMP-2 deficient mice showed an increased postnatal mortality rate which was associated with the development of a unilateral or bilateral hydronephrosis caused by an obstruction of the ureteropelvic junction.[39] An accumulation of lysosomes in the epithelial cells of the ureter adjacent to the ureteral lumen and a disturbed apical expression of uroplakin was observed, suggesting an impairment of membrane transport processes. Serious hearing deficiencies in LIMP-2 deficient animals was indicated by deficits in acoustic startle responses, in brainstem evoked auditory potentials and a reduced potassium concentration in the endolymph. LIMP-2 deficient mice suffer from a massive decline in spiral ganglia in the cochlea concomitant with a loss of the inner and outer hair cells. These pathological changes begin at the age of 3 months and are probably secondary to a degeneration of the stria vascularis. LIMP-2 deficient mice are also characterised by a peripheral demyelinating neuropathy. Demyelinisation was found to be associated with a massive loss of peripheral myelin proteins and an increased activity and expression of lysosomal proteins, highlighting a hitherto unknown role of the lysosomal compartment in the development of this myelination disorder.

Intracellular Trafficking of the Major Lysosomal Membrane Glycoproteins

LAMPs and LIMPs are mainly localised in the limiting membranes of lysosomes and late endosomes (Fig. 4). Small amounts are also found in early endosomal membranes[40] and the plasma membrane.[9,41] LAMP-1 and LAMP-2 are also present on the limiting membrane of late autophagic vacuoles.[27] The cell surface expression of LAMP-1 and/or LAMP-2 is increased during the activation of platelets and peripheral blood monocytes, as well as in cytotoxic T lymphocytes and in highly metastatic tumour cells.[42,43] By immunoelectron microscopy and biochemical methods, LAMP-1 and LAMP-2 are additionally found in the lysosomal/endosomal lumen, possibly associated with the internal membranes of lysosomes or endosomes.[44,45] The LAMP-2 mRNA undergoes alternative splicing which generates three isoforms with different transmembrane and cytosolic domains, referred to as LAMP-2a, b and c. LAMP2c has a predominantly lysosomal localisation, while higher levels of LAMP-2a and LAMP-2b are present

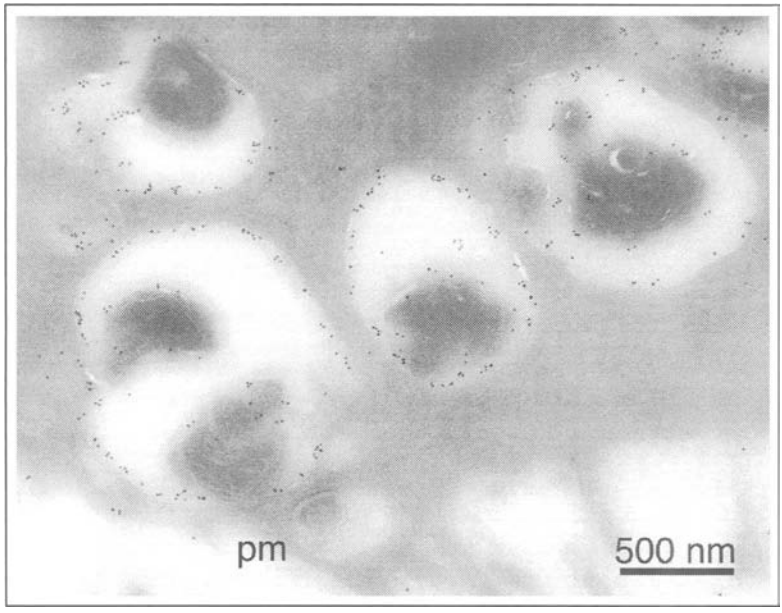

Figure 4. Lysosomal membrane proteins are primarily localised to the limiting membrane of lysosomes. Immunogold electronmicroscopy also reveals intralysosomal membrane structures with LAMP-1 staining. The cryosection was obtained from mouse hepatocytes and immunolabelled with anti-LAMP-1, followed by a secondary antibody coupled to 10 nm gold.

on the cell surface.[46] LAMPs and LIMPs are not static components of the limiting membrane; they are in dynamic equilibrium between lysosomes, endosomes and the plasma membrane.[47,48]

After synthesis LAMPs and LIMPs are transported from the TGN to endo/lysosomes mainly via an intracellular route without appearance on the cell surface.[4] LAMP-1 and LAMP-2 molecules in the TGN are packed into vesicles distinct from MPR and AP-1 adaptor containing clathrin-coated vesicles,[49] though some LAMP-1 may also be sorted into the latter vesicle type.[50] The lysosomal targeting depends on either a tyrosine-based (LAMP-1, LAMP-2, LIMP-1) or di-leucine-based (LIMP-2/LGP85) sorting signal in the cytoplasmic tail (refs. 51-53 and references therein). LAMP-1, LIMP-2/LGP85 and LIMP-1/CD63 have been shown to be targeted from the TGN to lysosomes via an intracellular route[54] that is dependent on the AP-3 adaptor complex.[53,55]

Less Abundant Integral Components of the Lysosomal Membrane

There are also less abundant lysosomal membrane proteins which are often expressed in a cell-type or tissue-specific manner (Table 1). The acidification of vacuolar compartments plays an important role in a variety of cellular processes, particularly membrane traffic processes. The conserved vacuolar proton pump (V-type H^+-ATPase; (Fig. 3) is located in the lysosomal membrane and couples the hydrolysis of ATP to the translocation of protons (reviewed in ref. 56). It consists of 13 different subunits and is responsible for the acidification of the lysosomal lumen. Eight of these subunits form a functional unit located on the cytosolic side, which is responsible for the ATP hydrolysis. The other five subunits form an integral membrane portion and mediate proton translocation. Knockout mice and human patients with mutations in an osteoclast specific proton pump subunit develop severe osteopetrosis.[57,58] It is possible that the lysosomal acidification is regulated by the expression of the tissue-specific isoforms of this proton pump. A general lack of lysosomal acidification probably leads to early embryonic lethality.

Table 1. Summary of components of the lysosomal membrane: topology, glycosylation, and evidence for lysosomal localisation

Common Name	Molecular Weight (Backbone)	Trans-membrane Domains	N-Glycosyl-ation Sites	Evidence for Lysosomal Localisation
LAMP-1	90-120 kDa (ca. 45 kDa)	1	17-20 N-Gly	endogenous staining
LAMP-2	95-120 kDa (ca. 46 kDa)	1	~ 17 N-Gly	endogenous staining
LIMP-1/CD63	ca. 40 KDa (ca. 26 kDa)	4	3 N-Gly	endogenous staining
LIMP-2/LGP85	74-85 kDa (ca. 53 kDa)	2	~ 11 N-Gly	endogenous staining
V-H^+-ATPase	SUa ca. 94 kDa SUd ca. 39 kDa SUC,C´ ca. 17 kDa SU C´´ ca. 20 kDa V1: SU A-H (soluble)	> 6 - 4 5	-	endogenous staining
Cystinosin	40-72 kDa (ca. 40 kDa)	7	7pN-Gly	endogenous staining
Sialin	55-80 kDa (ca. 55 kDa)	10-12	6 pN-Gly	endogenous staining
NPC1	140-240 kDa (ca.140 kDa)	13	23 pN-Gly	endogenous staining
CLN3	48-66 kDa (ca. 48 kDa)	6-8p	4 pN-Gly	overexpression studies
LALP70	70 kDa	2p	1-2 pN-Gly	overexpression studies
LAPTM4	26 kDa	4p	-	overexpression studies
ABC B9	84-100 kDa (ca. 84 kDa)	6-9p	4 pN-Gly	overexpression studies
ABC A2	ca. 270 kDa	12p	12 pN-Gly	overexpression studies
Nicastrin	80-150 kDa (ca. 78 kDa)	1-2	16 pN-Gly	endogenous staining
Endolyn/ CD164	22-60 kDa (22 kDa)	1-3p	9 pN-Gly	endogenous staining
TMEM9	22-30 kDa (ca. 22 kDa)	1p	3 pN-Gly	overexpression studies
LYAAT-1	52-70 kDa (52 kDa)	9-11p	5 pN-Gly	overexpression studies
DC-LAMP	45 kDa	1	2 pN-Gly	endogenous staining
P67	67 kDa (*Trypanosoma brucei*)	2p	14 pN-Gly	endogenous staining

Amino acids are translocated across the lysosomal membrane by a number of transporters (see also Chapter 7). Recently cystinosin (Fig. 3), the cystine transporter, has been identified and cloned. The gene encoding cystinosin, CTNS, is mutated in patients suffering from the lysosomal storage disease cystinosis, which primarily affects kidney function.[59,60] Cystinosin spans the membrane seven times. The existence of other lysosomal amino acid transporters is supported by the partially successful treatment of cystinosis patients with cysteamine, an aminothiol. This drug enters the lysosomal compartment through a specific but as yet unidentified transporter, reacts with the stored cystine to form a mixed disulfide which is subsequently able to leave the lysosome through an intact lysine transporter.[59] Recently a novel lysosomal transporter, LYAAT-1, that displays the functional characteristics of an H+/amino acid symporter has been identified. After overexpression in COS cells this protein mediated the lysosomal transport of neutral amino acids, such as γ-aminobutyric acid, L-alanine and L-proline. LYAAT-1 is predominantely localised in lysosomes of brain neurons.[2,61]

One monosaccharide transporter has been identified and linked to another lysosomal storage disease, known as sialic acid storage disease or Salla disease.[62] The main symptoms are hypotonia, cerebellar ataxia, atrophy and mental retardation. The responsible AST gene, which is mutated in these patients, encodes sialin which has 12 transmembrane domains (Fig. 3 and see Chapter 7). Apart from cystinosin and sialin, the existence of many other lysosomal membrane transporters involved in amino acid transport, mono- and oligosaccharide transport and oligopeptide transport, has been demonstrated, though their exact nature remains unknown. Recently a novel, highly conserved and ubiquitously expressed human transmembrane protein 9 (TM9EM9) that localises to late endosomes and lysosomes was identified. Although no hitherto known functional domains were described, a role in lysosomal transport processes has been suggested.[63]

LAPTM4α traverses the lysosomal membrane four times and is possibly involved in mediating the transport of nucleotides.[64] Recently, two putative ABC transporters were also cloned and, after transfection, shown to be localised in the lysosomal membrane.[65,66] NRAMP2 (natural resistance-associated macrophage protein 2), which is a divalent metal transporter, is an integral membrane glycoprotein with 12 transmembrane domains localised in late endosomes and lysosomes. NRAMP2 functions as an endosomal iron transporter in the transferrin cycle.[67]

In order to maintain proper cellular function, the amount and distribution of cholesterol residing within cellular membranes must be regulated. The NPC-1 protein with 13 transmembrane domains and a lysosome-targeting motif has received particular attention as mutations in the NPC gene result in another lysosomal storage pathology, known as Niemann-Pick type C disease. In patient fibroblasts, the pathological accumulation of cholesterol is apparently due to a failure in the mechanism responsible for the redistribution of cholesterol taken up by endocytosis of LDL[68,69] (see also Chapter 9).

In neuronal ceroid lipofuscinosis (NCL) or Batten disease, mutations in another integral lysosomal membrane protein CLN3 have been identified (The International Batten Disease Consortium). NCLs are autosomal recessive inherited storage disorders that represent the most common reason for progressive encephalopathy in children. The clinical features include psychomotoric retardation, progressive loss of vision and premature death.[70] Colocalisation with a synaptic vesicle marker suggests that CLN3 may be involved in synaptic vesicle transport.[71] It is also thought to function as a proton sensor, as human CLN3 can complement the CLN3-deficient yeast, *btn1*-Δ, in which the vacuolar pH is decreased in the early phase of growth.[72] Endolyn, like LAMP-1 and LAMP-2, is a highly glycosylated type I lysosomal and endosomal membrane protein of 78 kDa. It resembles sialomucin-like proteins and exhibits structural similarities to cytokine and growth factor family members.[73] A relatively high proportion of endolyn can also be detected on the plasma membrane, suggesting an indirect biosynthetic route to lysosomes and endosomes.

LALP70 (lysosomal apyrase-like protein of 70 kDa), which is almost identical to a Golgi UDPase, is a type III integral membrane protein located in a subfraction of lysosomal/

autophagic vacuoles[74] and is thought to play a role in nucleotide metabolism. The lysosomal membrane enzyme acetyl-CoA: alpha-glucosaminide N-acetyltransferase catalyses the transfer of an acetyl group from acetyl-CoA in the cytosol to terminal alpha-linked glucosamine residues of heparan sulfate in the lysosomal matrix.[75] A genetic deficiency of this membrane-bound enzyme leads to the severe lysosomal storage disease, Sanfilippo disease type C (MPS IIIC), with storage of nondigested heparan sulfate and heparin,as the catabolism of heparan sulphate and heparin cannot be completed.

DC-LAMP, a member of the LAMP family is a lysosomal membrane protein specifically expressed by human denditic cells upon activation. Sequence analyses revealed a 50% identity to LAMP-1. DC-LAMP is detected within MHC class II molecules-containing compartments just before translocation of the MHC class II-peptide complexes to the cell surface suggesting an involvement in this process.[76]

The precursor of lysosomal acid phosphatase (LAP) is another well-defined constituent of the lysosomal membrane. This hydrolase is transported from the endoplasmic reticulum to the lysosomes as a type I membrane glycoprotein. After arrival in the lysosome, the luminal domain of LAP, containing the active site of the enzyme, is slowly released into the lysosomal matrix by proteolytic processing.[51] LAP shares overlapping functions with the luminal tartrate-resistant lysosomal acid phosphatase, TRAP. Studies using knockout and double knockout mice suggested that both proteins are possibly involved in the dephosphorylation of osteopontin and bone metabolism.[77]

Perspectives

Although the major lysosomal membrane proteins have been known for some time, we are only beginning to reveal their functions. Clearly they are more than structural components, simply involved in maintaining the stability and integrity of the lysosomal limiting membrane. Mutations in mice and humans have revealed the more dynamic and unexpected roles of these proteins in lysosomal biogenesis and lysosomal/endosomal function. Subcellular proteomic analyses (see Chapter 11) will most likely uncover many hitherto unknown integral components of the lysosomal membrane and yield further information about how normal lysosomal membrane transport, stability and function are maintained.

Acknowledgements

The work presented in this review was supported by the Deutsche Forschungsgemeinschaft the Fonds der Chemischen Industrie and the Hensel-Stiftung, University Kiel. I am grateful to Eeva Liisa Eskelinen for critically reading the manuscript.

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CHAPTER 5

Lysosomal Proteases:
Revival of the Sleeping Beauty

Klaudia Brix*

Abstract

Lysosomal proteases belong to the aspartic, cysteine, or serine proteinase families of hydrolytic enzymes. They are expressed ubiquitously, and in a tissue- or cell type-specific manner. Although we still call them lysosomal proteases, the enzymes are usually detected within all vesicles of the endocytic pathway. In specific cell types, they might even become secreted and might fulfill important tasks in the direct pericellular surrounding. Functions of lysosomal proteases comprise bulk protein degradation within lysosomes, antigen processing within early endosomes, proprotein processing at unexpected locations such as secretory vesicles, prohormone processing and degradation of matrix constituents in the extracellular space, and, most recently, lysosomal proteases have been proposed to contribute to the initiation of apoptotic processes within the cytosol. Many of these functions were determined through the use of cathepsin-deficient mice which also demonstrated the redundancy of some cathepsins, i.e., those belonging to the cysteine proteinases. Challenges for future research on lysosomal proteases are to uncover more of their in vivo substrates and to clarify where and which of the many enzymes are essential for the maintenance of vital functions of cells, tissues, or organs.

Introduction

Lysosomal proteolytic enzymes catalyze the hydrolysis of proteins. Only few of the proteinases work as amino- or carboxypeptidases, while most are endopeptidases preferably cleaving peptide bonds within a polypeptide chain rather than at its ends. Accordingly, the name cathepsin was coined which combines the Greek verbs to digest, i.e., cata-, and to boil, i.e., hepsein. Originally, the term cathepsin was meant to be reserved for endopeptidases. However, it turned out that cathepsin B1 which, to follow this convention, was explicitly named as such,[1] is both, an endo- and an exopeptidase. In the late 1990s, a more recent nomenclature discussion was started by members of the International Proteolysis Society (www.protease.org). Due to the progress in genome sequencing, the scientific community was afraid of shortly running out of letters of the alphabet to be added to the term cathepsin in order to name newly identified enzymes. However, no better nomenclature system was found, and, hence, we still stick to the term cathepsin, but now, we add numbers instead of letters.

An excellent reference and a classification of lysosomal proteases can be found in the MEROPS database (merops.sanger.ac.uk).[2] Metalloproteinases, with the exception of lysosomal dipeptidase I, proteases with unknown mechanism of proteolytic cleavage, and the most recently discovered threonine proteases are rarely found within lysosomes. Mainly, lysosomal proteases are members of the aspartic, cysteine, or serine proteinase families of proteolytic enzymes.

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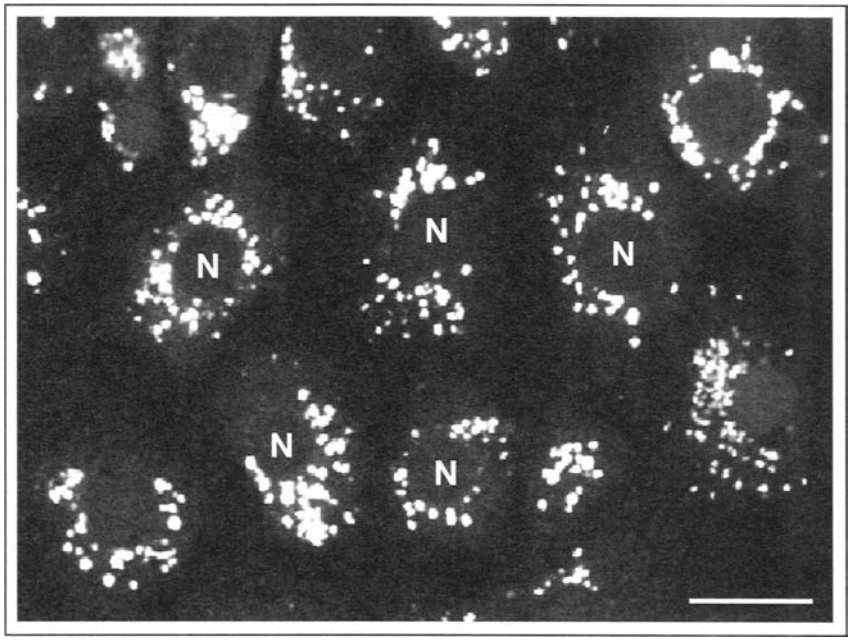

Figure 1. Cathepsin B, a cysteine proteinase, is expressed in lysosomes of porcine thyroid epithelial cells. Note, the abundance of cathepsin B-containing vesicles in the perinuclear region; this distribution is typical for lysosomes. Immunofluorescence staining after formaldehyde-fixation and membrane permeabilisation. Micrograph was taken with a confocal laser scanning microscope, N = nuclei, bar = 20 µm.

Expression and Distribution of Lysosomal Proteases

Many lysosomal proteases are ubiquitously expressed, like the aspartic proteinase cathepsin D or the cysteine proteinases cathepsins B, H and L (Fig. 1); others exhibit a more tissue-specific expression pattern. Different cell types, the same cell type but in different physiological conditions, or even the endocytic compartments of one and the same cell may exhibit variable patterns of expression of lysosomal proteases (Fig. 2). Furthermore, the expression of lysosomal proteases, very much like that of many other cellular proteins, is regulated in order to enable a cell to respond to changing physiological situations. Even when looking at the same cell type in a given physiological condition, e.g., an antigen-presenting cell encountering a pathogen, the proteolytic activities of different lysosomal proteases might be of variable significance within distinct endocytic compartments such as early endosomes, late endosomes, and lysosomes.[3] Cathepsin S, to name a representative example, might well be present and active in the processing of antigen in the endosome of an antigen-presenting cell, but it might be of less importance in the degradation processes within the lysosomes of that same cell, whereas exactly the opposite might be true for cathepsin B. Such variations in the importance of different proteases for proteolytic processes within endocytic compartments might be explained by their different pH-optima for substrate cleavage or by the differences in their stability at different pH-conditions.[4]

Furthermore, cells might specifically up-regulate one set of enzymes whereas another set might be expressed at lower levels in disease situations such as cancer.[5] Above it, different isoforms of lysosomal proteases have been described which are more abundant in, but not limited to cancer cells.[6] For example, alternatively spliced mRNA's for cathepsin B exist which lack the sequences encoding for the signal peptide.[7] As a consequence, such isoforms of cathepsins might even be present within the nucleus. Usually, however, the signal peptide

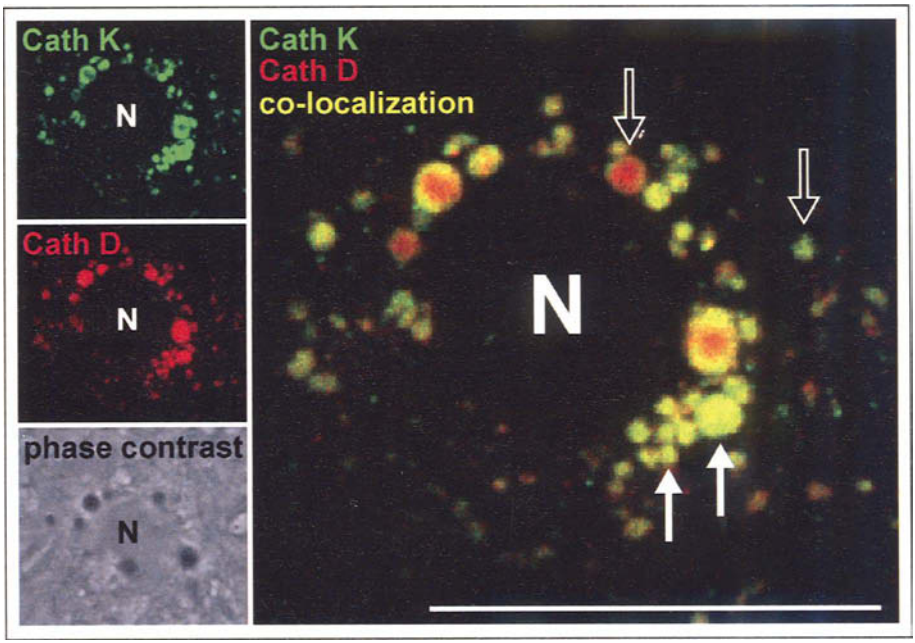

Figure 2. Expression of the aspartic lysosomal protease cathepsin D (red) and of the lysosomal cysteine protease cathepsin K (green) in porcine thyroid epithelial cells. Both enzymes are mainly colocalised within the same vesicles as indicated by the yellow color (arrows). However, immunolabeling also showed that some compartments of the endocytic pathway contained only one of the two lysosomal proteases (open arrows). Immunofluorescence staining after formaldehyde-fixation and membrane permeabilisation. Micrographs were taken with a confocal laser scanning microscope, N = nuclei, bar = 20 µm.

is responsible for targeting of cathepsins for entry into the lumen of the endoplasmic reticulum from where they are transported as inactive precursor forms to the Golgi (Fig. 3), and to late endosomes and lysosomes for proteolytic maturation.[8] For a more detailed discussion of the transport pathways of lysosomal enzymes, see Chapter 2.

Importance of the in Situ Determination of Proteolytic Activities

The term "lysosomal proteases" is somewhat misleading, because most, if not all of these enzymes are detected within all compartments of the endocytic pathway, i.e., endocytic vesicles, early endosomes, late endosomes, autophagic vacuoles, and lysosomes. In specific cell types or tissues, the lysosomal proteases are even secreted and perform important tasks at the cell surface or in the pericellular environment (Figs. 4, 5).[4,5,8,9] For a comprehensive discussion on external lysosomal enzymes, see Chapters 12 and 13.

As already mentioned above, several isoforms of specific lysosomal proteases might be expressed by the same cell, with few of them being present at cellular locations which are as unusual and unexpected for a "lysosomal protease" as the cytosol or the nucleus.[10,11]

Enzyme cytochemical staining techniques proved that the so-called lysosomal proteases are proteolytically active at other locations than simply within the compartments of the endocytic pathway.[12-16] Up until now, most information that we gain by using these approaches tells us whether a given protease is proteolytically active, for example, within the endosomes and at the cell surface in addition to the proteases' expected lysosomal activity. However, the precise determination of the extent of proteolytic activity at a particular location is by far more complicated to deduce from such experiments, because one would

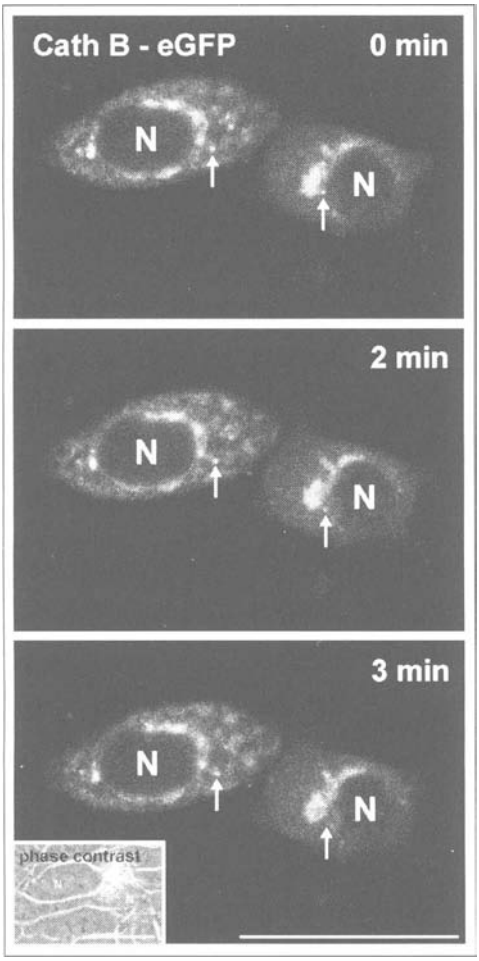

Figure 3. Transport of lysosomal cysteine proteinases in rat thyroid epithelial cells as viewed by confocal laser scanning microscopy after expression of cathepsin B-enhanced green fluorescent protein (Cath B-eGFP). Accumulation of the enzyme in the trans-Golgi region at 10°C, and formation and budding of Cath B-eGFP-containing vesicles (arrows) after rewarming to the transport-permissive temperature of 37°C for the indicated time intervals. N = nuclei, bars = 50 μm.

need to know the exact pH- and redox-conditions of the compartments and, most importantly, the natural substrates instead of synthetic substrates would have to be used to quantitatively determine the proteolytic potency of a particular enzyme at a given cellular location.

The manifold of lysosomal proteases and their complex patterns of differential expression had long been over-looked, because it seemed clear that the main task for lysosomal proteases is the gross degradation of proteins internalized through endocytosis. But now, as we learn more about which and where lysosomal proteases are expressed, we are challenged in predicting and proving the functions of individual lysosomal proteases, i.e., we have to wake up the Sleeping Beauty. A substantial step forward towards this goal was the technique of targeted gene knock-out.

Functions of Lysosomal Proteases

Mice with a deficiency in cathepsin A, i.e., a lysosomal serine protease, showed a phenotype similar to human galactosialidosis, a lysosomal storage disease.[17] This phenotype, however, was due to the so-called protective function of cathepsin A, i.e., to its ability to stabilize lysosomal glycosidases through the formation of a multienzyme complex, rather than to its catalytic activity. The physiological function of cathepsin A which is associated with its proteolytic activity, has been suggested to be the degradation of lamp2a, a lysosomal membrane glycoprotein that

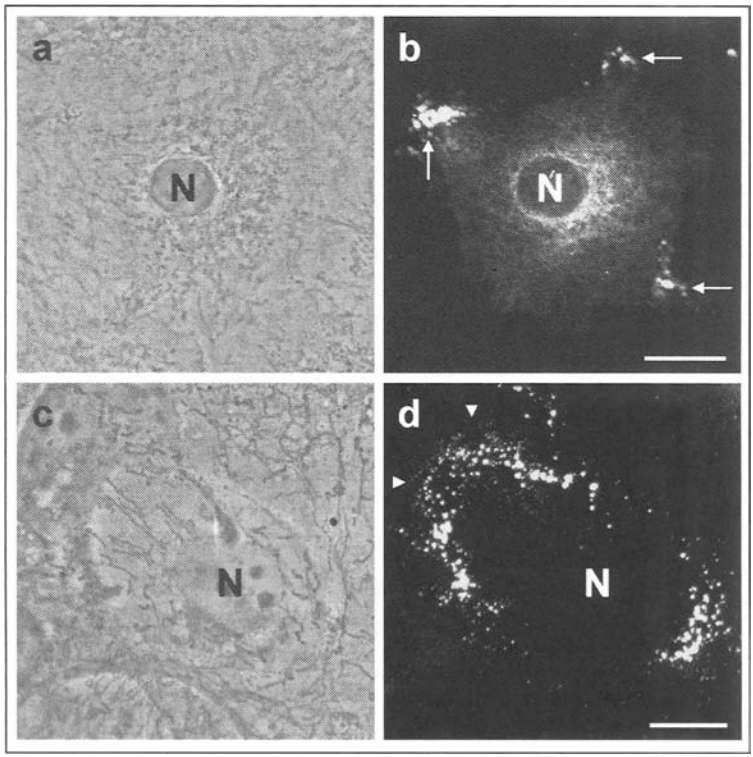

Figure 4. Cathepsin K is expressed by porcine thyroid epithelial cells, see also Figure 2 and reference 42. Note, that cathepsin K-containing vesicles are located in the cell periphery (a, b, arrows), and cathepsin K is also secreted into the pericellular space (c, d, arrowheads). Immunofluorescence staining after formaldehyde-fixation (c-d) and membrane permeabilisation (a-b). Micrographs were taken with a confocal laser scanning microscope, N = nuclei, bars = 10 μm.

serves as a receptor for chaperone-mediated autophagy.[18] Another lysosomal serine protease has been knocked-out in mice, i.e., cathepsin G, resulting in mice without an overt phenotype.[19,20]

Asparaginyl endopeptidase (AEP), a lysosomal cysteine proteinase that was originally discovered as the plant vacuolar processing enzyme legumain, was suspected to be of vital importance in the mouse, because it mediates the biosynthetic processing of the ubiquitously expressed cathepsins B, H, and L which also belong to the family of lysosomal cysteine proteinases. Strikingly, however, the AEP/legumain-deficient mice were normally born and they were fertile.[21] Since AEP seems to mediate the proteolytic processing of the single-chain form into the two-chain form of the cathepsins B, H, and L,[21] and because both of these molecular forms are in principal proteolytically active, the relatively mild phenotype of the AEP-deficient mice can be explained.

Of the large group of lysosomal cysteine proteases, cathepsin B is ubiquitously expressed and it was one of the first being knocked out. However, the targeted deletion of the cathepsin B gene resulted in mice in which the overall phenotype was not altered.[22] Cathepsin D, a ubiquitously expressed aspartic lysosomal protease, on the other hand, proved to be essential for the survival of mice, because its deficiency led to death due to anorexia about three weeks after birth.[23] Cathepsin D is essential in that it regulates cell growth and/or tissue homeostasis through mediating the limited proteolysis of regulatory proteins.[23] More recently, it was observed that cathepsin D-deficient mice also have a phenotype that resembles neuronal ceroid lipofuscinosis,[24] which is covered in Chapter 8.

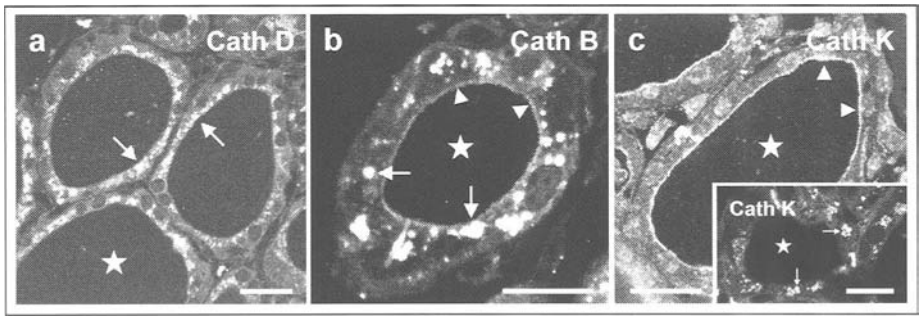

Figure 5. Expression of lysosomal proteases at unexpected locations in the mouse thyroid. The aspartic protease cathepsin D is mainly located within lysosomes of epithelial cells of thyroid follicles (a, arrows). Although the cysteine proteinases cathepsins B (b) and K (c) are members of the family of lysosomal proteases, they often become secreted into the thyroid follicle lumen (asterisks), and reassociate with the apical surface of thyroid epithelial cells (b and c, arrowheads). The inset in c shows cathepsin K expression within lysosomes (arrows). The lysosomal cysteine proteinases cathepsins B, K, and L start to process thyroglobulin extracellularly; cathepsins K and L are essential for thyroxine liberation.[37] Micrographs were taken with a confocal laser scanning microscope after immunolabeling of cryosections of formaldehyde-fixed mouse thyroids. Bars = 20 μm.

Both cathepsins B and D, had long been candidates for mediating endosomal antigen processing during MHC class II-mediated antigen presentation. However, cathepsins B and D were both shown to be dispensable for antigen processing.[22] Instead, cathepsins L and S are important for this process although the two cysteine proteinases seem to share the burden in various antigen-presenting cells.[3,25,26] Cathepsin F, another member of the cysteine proteinase family, seems to be responsible for invariant chain processing in macrophages, whereas this important proteolytic processing task enabling peptide loading onto MHC-class II is mediated by the cysteine proteinase cathepsin S in splenocytes, dendritic cells and B cells, and by the cathepsin L-relative cathepsin V in thymic epithelial cells.[26-28] In addition, AEP/legumain might be of importance for the initial steps of invariant chain processing in dendritic cells and in B cells.[29] Hence, a number of lysosomal cysteine proteinases contribute to a biological function as important as antigen processing and presentation.[3,25,26]

The observation that quite a few of the cathepsin-deficient mice showed mild phenotypes and that the different albeit related members of the cysteine proteinase family might be differentially important in various cell types might look confusing at the first glance. It does, however, clearly demonstrate one of the major lessons learned from cathepsin-deficient mice. This is to say that in particular the largest group of lysosomal proteases, i.e., the family of lysosomal cysteine proteinases, shows an astonishingly high degree of redundancy.[25,30] More than 10 different enzymes have been identified that in the mouse system, and according to sequence similarities, can be further classified into subfamilies. A prominent example of such a subfamily is the group of placental cysteine proteinases which were discovered in mice and rats but seems to be absent or less abundant in human or rabbit placenta.[31] However, lysosomal cysteine proteinases provide cells of many organisms with a comprehensive set of redundant proteolytic activities.[25,32] Assuming that one of these enzymes might be defect due to genetic mutations, or, as in the case of targeted gene knock-out, might be completely missing, the out-come of such alterations might be less dramatic than expected because there are a couple of similar enzymes that could, in principle, do the same job.

In contrast to the cathepsin B-deficient mouse that did not have an overt phenotype, some other deletions of cysteine proteinase genes clearly proved that specific proteases are important for a variety of biological functions. One such example is the cathepsin L-deficient mouse.[33] Cathepsin L-deficiency did not result in lethality, but these mice showed a couple of organ-specific

phenotypes demonstrating that cathepsin L has important functions in the heart, skin, thymus, and in the thyroid.[26,33-37] Another example of such a "successful" gene knock-out of a lysosomal cysteine protease was the cathepsin K-deficient mouse which exhibited severely impaired bone matrix resorption resulting in osteopetrosis.[38] Indeed, the cathepsin K-deficient mouse was accepted as a valuable animal model for the rare human disorder *Pycnodysostosis* in which various mutations lead to the expression of nonfunctional cathepsin K resulting in short stature and bone malformation in patients suffering from this disease.[39] Because cathepsin K was believed to be expressed almost exclusively by osteoclasts and in ovary,[40] the enzyme was soon recognized as a suitable, if not ideal target for the development of therapeutic drugs to treat osteoporosis, i.e., a common disease of the elderly of Western populations, which results in enhanced bone matrix degradation and which leads to a decrease in bone density. Later, it became obvious that cathepsin K is also expressed by a variety of non-osteoclast cells including epithelial cells of the lung,[41] and the thyroid (Figs. 2, 4, 5).[42] As one would expect from a broader expression pattern, cathepsin K's function, at least in the mouse, is not restricted to bone matrix degradation, rather cathepsin K, together with cathepsin L, also mediates thyroxine liberation through proteolytic degradation of thyroglobulin, the glycoprotein prohormone of the thyroid.[4,37] It has to be further added that cathepsin K might be the main enzyme responsible for degradation of long bones, however, it does not mediate remodeling of all bone structures of the body.[38] Therefore, cathepsin K still is an excellent example for the identification of a putative drug target for therapeutic intervention, however, future has to show whether such treatments would interfere with cathepsin K's function in tissues other than bone.

Specificity of Lysosomal Proteases-How to Find the in Vivo Substrates

Although the cathepsin-deficient mice were very successful in determining some functions of lysosomal proteases, the unexpected mild phenotypes in many of these mice pointed to the problem of redundancy as discussed above. Redundancy always raises the question as to whether single members of the redundant system are essential in that they perform specific tasks, or whether they might be present simply to serve as a back-up. Above it, a redundant group of enzymes with overlapping functions is often viewed as a non-selective system. However, what is called non-specificity or non-selectivity of lysosomal proteases might simply reflect the lack of knowledge of the natural substrates of the enzymes. Therefore, the approach of challenging mice with a deficiency in one of the lysosomal proteases which originally showed no or only a mild phenotype is one way to encircle this problem. Another approach is to cross mice with single protease deficiencies, and hope for a phenotype in double- or triple-deficient animals. Both approaches have been successful in the identification of some in vivo substrates of lysosomal proteases.

Natural Substrates of Lysosomal Proteases at Unexpected Locations

Crossing cathepsin B- with cathepsin L-deficient mice resulted in an early-onset brain atrophy which led to the death of the double-deficient animals approximately four weeks after birth.[43] From these observations, it can be deduced that very drastic measures, i.e., deficiencies in two of the three ubiquitously expressed lysosomal cysteine proteinases or deficiency in the one ubiquitously expressed aspartic lysosomal protease,[43,23] are needed to interfere with the functions of lysosomal proteases in such a way that it results in death of the animals in an early postnatal stage. The results of these studies do not allow, however, to conclude on the specific substrates of the lysosomal proteases.

When cathepsin B-deficient mice were challenged through an experimentally induced acute pancreatitis, it became evident that cathepsin B is essential for the activation of trypsinogen.[44] A thyroid phenotype with systemically reduced levels of thyroxine in the blood was observed in mice with a double-deficiency in cathepsins K and L (see also Fig. 5).[37] In addition, it was shown that the prohormone thyroglobulin is processed not only by cathepsins K and L but also by cathepsin B, and that this proteolytic processing occurs not only within

endosomes and lysosomes, but begins even extracellularly.[37] Another prohormone-processing function of cathepsin L has been observed in vesicles of the secretory pathway, again, at an unexpected location.[45] Hence, in all these cases the in vivo substrates of the lysosomal proteases were identified and the function of the lysosomal cysteine proteinases obviously was the limited proteolytic processing of large precursor proteins. The locations of proteolytic processing were, however, rather unexpected.

Limited proteolysis of as yet unidentified substrates such as growth factors or their receptors was also proposed to explain the function of lysosomal proteases in the regulation and control of tissue homeostasis. The aspartic protease cathepsin D seems to be essential for the survival of ileal mucosa cells.[23] Cathepsin L of the cysteine proteases is important for survival of thyroid epithelial cells,[37] whereas its deficiency induces hyperproliferation of epidermal keratinocytes.[33,36] These results exemplify that cathepsins are involved in the regulation of cell survival and cell death. Furthermore, cathepsin B was proved to mediate TNF-alpha-induced hepatocyte apoptosis.[46]

The notion of cathepsin B's involvement in hepatocyte apoptosis and the observation that several cytosolic or mitochondrial apoptotic factors are cleaved by lysosomal proteases, prompted an entirely new field of activity, both, for the proteases and for the researchers. If lysosomal enzymes indeed contributed to the onset of apoptotic cell death,[10,11] they must reach the cytosol either through transport out of the lysosomes or isoforms of the enzymes must be directly translated from free ribosomes. These questions remain unanswered at present. It is, however, quite clear that cathepsins of the cysteine proteinase class are involved in apoptosis of various cell types.

Apoptotic cell death of neurons and neuronal atrophy in various brain regions is a consequence of cystatin B-deficiency.[47] Cystatin B is an endogenous inhibitor of a variety of lysosomal cysteine proteinases such as cathepsins B, H, L, and S.[48] The cystatin B-deficient mouse serves as an animal model for progressive myoclonus epilepsy of the Unverricht-Lundborg type (EPM1) which is a recessively inherited neurodegenerative disease.[47] When cystatin B-deficient mice were crossed with mice with deficiencies in either cathepsins B, L, or S, it was shown that cathepsin B contributed to the pathogenesis of the disease.[49] However, similarly to what is discussed above, the redundancy problem appeared here, again, because deficiency of cathepsin B did not fully reduce the amount of cerebellar granule cell apoptosis.[49]

Apart from the fact that we have to clarify how cathepsins could reach the cytosol, if they really came from the vesicles of the endocytic pathway, it is important to identify the substrate that is cleaved by cathepsin B and by the as yet unidentified protease of the cysteine proteinase family that contributes to the apoptosis-initiating activity.

Perspectives

The most important task for the future is to solve the problem of redundancy. Although the knock-out mice phenotypes were not as striking and drastic as expected, and although the up-regulation of other enzymes with overlapping functions might be an explanation for mild phenotypes, only very little is known about the effects of deficiency in one protease on the expression of others,[37] and, even more important, on the level of proteolytic activity of the entire set of lysosomal proteases.

Clearly, in the post-genomic era, we should be able to solve the problem of which and how much protease is expressed by a specific cell or at a particular location within a cell; see also Chapter 11. Most recent developments towards the selective targeting of specific proteases with active site-directed probes allow the enzyme activity profiling of distinct subproteomes.[50-54] Profiling of proteolytic activities in an array-based, proteome-wide,[53] and, thus, in a more global approach may enable us to assess the complexity of the system of lysosomal proteases.

Hence, the light on the horizon is there, and we will soon be able to quantify the extent of proteolytic activities in situ and in vivo. However, the prediction is, now that we were successful in reviving the Sleeping Beauty, it may well take a little longer to fully understand the proteases which we still refer to as "lysosomal proteases".

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CHAPTER 6

Lysosomal Storage Disorders

Ole Kristian Greiner-Tollersrud and Thomas Berg*

Introduction

The lysosomal storage disorders (LSD) are a group of about 50 diseases that are characterised by an accumulation of waste products in the lysosomes, resulting in the formation of large intracellular vacuoles (Fig. 1).

Many of the diseases that we now know as lysosomal storage disorders, were first described long before the discovery of the lysosome in 1955 by de Duve.[1] As the structure and function of this organelle was defined and the different lysosomal proteins identified, the concept of lysosomal storage disorders evolved. The last decade has witnessed major advances in our understanding of the clinical, biochemical and genetical aspects of lysosomal storage diseases.

Although individually rare the lysosomal storage disorders as a group have a frequency of about 1/8000 live births,[2,3] making this disease group a major challenge for the health care system. As an increasing number of patients with milder forms are being identified, the current figures may underestimate the actual frequencies of lysosomal storage disorders.

The group of lysosomal storage disorders is usually caused by the lack of a hydrolase, its activator or a transporter causing accumulation of specific substrates in the lysosomes for each disorder type. Recently defects of the vesicular transport in the endosomal/lysosomal system have been reported to cause phenotypes similar to lysosomal storage disorders, for example mucolipidosis type IV.[4] This group of deficiencies includes the neuronal ceroid lipofuscinoses and a growing number of pigmentary disorders, and the elucidation of the function of these proteins will in a fundamental way increase our understanding on how the vesicular trafficking in the cell is regulated. The storage diseases are inherited in an autosomal-recessive fashion, except Fabry disease,[5] Hunter disease (MPS II)[6] and Danon disease[7] that are all X-linked recessive. Certain disorders are more prevalent in certain geographic areas or among those of a particular ethnicity. For example the majority of patients with aspartylglucosaminuria[8] and Salla disease[9] are from Finland. In a similar fashion Gaucher disease[10] and Tay-Sachs disease[11] are almost 100 times more prevalent in Ashkenazi Jewish decent that in the general population.

The lysosomal storage diseases have a broad spectrum of clinical phenotypes. In addition the age of onset, severity of symptoms and central nervous system manifestation can vary markedly within a single disorder type. Several lysosomal storage disorders such as for example Gaucher disease,[12] Tay Sachs disease,[13] Pompe disease,[14] beta-galactosidase deficiency[15] have infantile, juvenile and adult forms.

The severity of a lysosomal storage disorder type will depend partially on the type of accumulating waste product. For example in beta-mannosidosis the accumulating product in affected goats and cows are the trisaccharide ManGlcNAcGlcNAc causing severe brain disease and early death,[16,17] while in affected humans that instead accumulate the disaccharide ManGlcNAc the disease phenotype is very mild.[18] The severity will also depend on which cells or tissues that accumulate the waste products. Other factors that affect the disease outcome are

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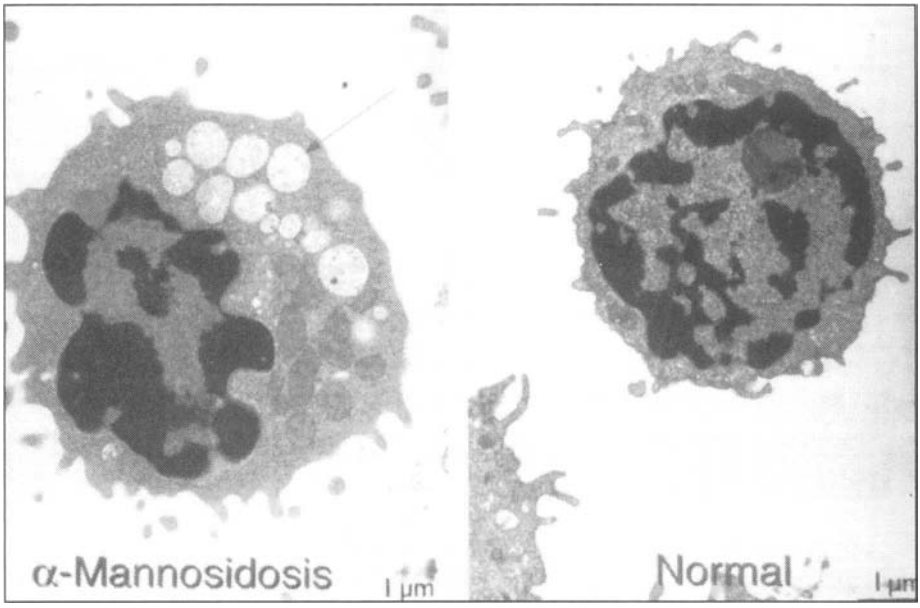

Figure 1. Enlarged vacuoles in a lysosomal storage disease. The electrom microscopy picture of a leukocyte cell from a patient affected with alpha-mannosidosis was kindly provided by Dr. Dag Malm.

the genetic background and environmental influence. The cells and tissues have certain thresholds of enzymatic activities below which clinical manifestations occur. This is probably the reason how an infantile and a juvenile/adult form of the same disease can affect different tissues, as for example beta-galactosidase-deficiency, which is named GM1-gangliosidosis in its infantile form causing severe brain disease, while in the juvenile/adult form it is named MPS IV since the phenotype is reminiscent of mucopolysaccharidosis without brain involvement.[15]

Most of the patients with a lysosomal storage disorder are born apparently healthy and the symptoms develop progressively. The speed and severity of the evolving symptoms depend on many factors as discussed above. Important from a therapeutic point of view is that lysosomal proteins added to the extracellular space will reach the lysosomes, usually via lectins on the plasma membrane as the mannose 6-phosphate receptor and the mannose receptor.[19,20] This concept is known as enzyme replacement therapy, and was first utilised successfully on patients with a mild form of Gaucher disease in the 1980s. The recombinant glucocerebrosidase was available for treatment in 1990.[21,22] Since then enzyme replacement therapy is a reality for Fabry disease[23] and Hurler disease (MPS 1).[24] Clinical trials with recombinant human enzymes are ongoing in Pompe disease,[25,26] MPS II[27] and MPS VI.[28] Enzyme replacement trials in several knock out mouse models, as for example acid sphingomyelinase deficiency (Niemann Pick disease)[29] and lysosomal α-mannosidase deficiency (α-mannosidosis)[30] have proven promising ensuring that clinical trials on new diseases are about to begin. Enzyme replacement by bone marrow transplantation continues to be effective in nonneuropathic Gaucher disease and in some forms of mucopolysaccharidosis[31] but it has high morbidity and mortality that limits its use in lysosomal storage disorders. Gene therapy has not been sufficiently developed to merit clinical trials, but studies on animal models of lysosomal storage disorders[32] have shown that it may become an alternative therapy as soon as the safety has been documented.[33] Recently, however, the planning of phase 1 clinical trials of Batten disease using adeno associated vectors was reported.[34] Another therapeutic approach is to decrease the access of substrate (substrate deprivation therapy). Recently an inhibitor of glucose transferase, N-butyldeoxynojirimycin, was shown

effective in treatment of nonneuronopathic Gaucher disease.[35] It is hope that within short future up to 50% of the lysosomal storage diseases may become treated by enzyme replacement. The targeting of enzyme drugs to all the cells that need them is still a major problem, especially through the blood-brain barrier. The success of therapy is dependent on an early intervention. The combination of new therapies and the combined frequency of lysosomal disorders of 1/8000 live births should make a newborn screening programme justifiable. Studies are continuing on finding markers that can be used in such a screening programme.[36]

The Lysosomal Storage Disorders

The lysosomal storage disorders were divided into the following 5 groups:

1. Defects in glycan degradation
2. Defects in lipid degradation
3. Defects in protein degradation
4. Defects in lysosomal transporters
5. Defects in lysosomal trafficking

Table 1 lists 50 lysosomal storage disorders belonging to these groups.

Defects in Glycan Degradation

The most common group of lysosomal storage disorders, represented by about 30 diseases, results from defects of glycan degradation. The group can be divided into the following four subgroups:

a. Defects in glycprotein degradation
b. Defects in glycolipid degradation
c. Defects in glycosaminoglycan degradation
d. Defect in glycogen degradation

Defects in Glycoprotein Degradation (Glycoproteinoses)

Figure 2 depicts how the lysosomal exoglycosidases acts in sequence[37] to cleave off monosaccharides from a complex type N-glycan. The hydrolases 5 ($\alpha^{1\text{-}6}$mannosidase) and C (chitobiase) are probably not required for the complete degradation as the glycosidic bonds cleaved by these also are cleaved by other lysosomal enzymes (Fig. 2). Indeed, no lysosomal disorder involving these enzymes have been reported. The deficiencies of hydrolase 3 (β-hexosaminidase) and hydrolase 2 (β-galactosidase) cause defects in glycoprotein-, glycosaminoglycan- and glycolipid-degradation. As the defects of glycolipid degradation cause the most severe clinical phenotypes, both of these enzyme deficiencies are discussed under part b). The deficiencies of hydrolase 6 (β-mannosidase) and hydrolase 7 (α-N-acetylgalactosaminidase (α-galactosidase B)) are both extremely rare and exhibit large variations in clinical severities from no apparent clinical symptoms to severe brain disease.[38,39] However, the combination of few patients, consanguineous parents and large variation in clinical symptoms makes it difficult to assess the consequences of these two diseases in humans. Possibly the severe clinical phenotypes reported in some patients are caused by other factors, and that in the absence of these the clinical symptoms are mild and the diseases thus possibly underdiagnosed.[18,40]

The deficiency of hydrolase 1 (sialidase) may be caused both by mutations in the sialidase gene [38] and in the gene encoding cathepsin A.[41] Cathepsin A-deficiency causes combined sialidase and β-galactosidase deficiency due to its function in stabilising these two hydrolases.[41] The early infantile forms cause mental retardation, dysostis multiplex, hepatosplenomegaly and early death. The clinical severities vary in continuum to the mild forms with normal life span and mild mental retardation.[38,41] The deficiency of hydrolase A (α-fucosidase) does not normally cause hepatosplenomegaly and the typical symptoms are mental retardation, recurrent infections, growth retardation and dysostis multiplex. The clinical symptoms vary in continuum from the severe form with death before age of 10 and mild form with life span into adulthood.[38] The deficiency of hydrolase 4 (lysosomal α-mannosidase) and hydrolase B

Table 1. Lysosomal storage disorders

Protein Defect	Disease	OMIM	Chromosomal Localization
DEFECTS IN GLYCAN DEGRADATION			
Defects in glycoprotein degradation			
α-Sialidase	Sialidosis	608272	6p21.3
Galactosialidosis	Cathepsin A	256540	20q13.1
α-Mannosidase	α-Mannosidosis	248500	19q12
β-Mannosidase	β-Mannosidosis	248510	4q22
Glycosylasparaginase	Aspartylglucosaminuria	208400	4q32
α-Fucosidase	Fucosidosis	230000	1q34
α-N-Acetylglucosaminidase	Schindler	104170	22q13.1
Defects in glycolipid degradation			
A. GM1 Ganglioside			
β-Galactosidase	GM1 gangliosidosis / MPS IVB	230500	3p21.33
β-Hexosaminidase α-subunit	GM2-gangliosidosis (Tay-Sachs)	606869	15q23
β-Hexosaminidase β-subunit	GM2-gangliosidosis (Sandhoff)	606873	5q13
GM2 activator protein	GM2 gangliosidosis	272750	5q31
Glucocerebrosidase	Gaucher disease	606463	1q21
Saposin C	Gaucher disease	176801	10q22.1
B. Defects in the degradation of sulfatide			
Arylsulfatase A	Metachromatic leukodystrophy	607574	2q13.31
Saposin B	Metachromatic leukodystrophy	176801	10q22.1
Formyl-Glycin generating enzyme	Multiple sulfatase deficiency	607939	3p26
β-Galactosylceramidase	Globoid cell leukodystrophy (Krabbe)	606890	14q.31
C. Defects in degradation of globotriaosylceramide			
α-Galactosidase A	Fabry	301500	Xq22.1
Defects in degradation of Glycosaminoglycan (Mucopolysaccharidoses)			
A. Degradation of heparan sulphate			
Iduronate sulfatase	MPS II (Hunter)	309900	Xq28
α-Iduronidase	MPS 1 (Hurler, Scheie)	607015	4p16.3
Heparan N-sulfatase	MPS IIIa (Sanfilippo A)	252900	17q25.3
Acetyl-CoA transferase	MPS IIIc (Sanfilippo C)	252930	14
N-acetyl glucosaminidase	MPS IIIb (Sanfilippo B)	252910	17q21
β-glucuronidase	MPS VII (Sly)	253220	7q21.11
N-acetyl glucosamine 6-sulfatase	MPS IIId (Sanfilippo D)	252940	12q14
Degradation of other mucopolysaccharides			
N-Acetylgalactosamine 4-sulfatase	MPS VI	253200	5q11-13
Galactose 6-sulfatase	MPS IVA (Morquio A)	253000	16q24.3
Hyaluronidase	MPS IX	601492	3p21.3
Defects in degradation of glycogen			
α-Glucosidase	Pompe	232300	17q25

Table continued on next page

Table 1. Continued

Protein Defect	Disease	OMIM	Chromosomal Localization
DEFECTS IN LIPID DEGRADATION			
Defects in degradation of sphingomyelin			
Acid sphingomyelinase	Niemann Pick type A and B	607808	11p15.2
Acid ceramidase	Farber lipogranulomatosis	228000	8q22
Defects in degradation of triglycerides and cholesteryls ester			
Acid lipase	Wolman and cholesteryl ester storage disease	278000	10q23.2
DEFECTS IN PROTEIN DEGRADATION			
Cathepsin K	Pycnodystostosis	601105	1q26
Tripeptidyl peptidase	Ceroide lipofuscinosis 2	607998	11q15.5
Palmitoyl-protein thioesterase	Ceroide lipofuscinosis 1	600722	1p32
DEFECTS IN LYSOSOMAL TRANSPORTERS			
Cystinosin (cystin transport)	Cystinosis	606272	17p13
Sialin (sialic acid transport)	Salla disease	604322	6q14
DEFECTS IN LYSOSOMAL TRAFFICKING PROTEINS			
UDP-N-acetylglucosamine Phosphotransferase γ-subunit	Mucolipidosis III (I-cell)	607838	16
Mucolipin-1(cation channel)	Mucolipidosis IV	605248	19p13
LAMP-2	Danon	309060	Xq24
NPC1	Niemann Pick type C	607623	11q11-12
CLN3	Ceroid lipofuscinosis	607072	16p12.1
CLN 6	Ceroid lipofuscinosis 6	606725	15q21-23
CLN 8	Ceroid lipofuscinosis 8	607837	8pter-p22
LYST	Chediak-Higashi	606897	1q42
MYOV	Griscelli Type 1	160777	15q21
RAB27A	Griscelli Type 2	603868	15q21
Melanophilin	Griscelli Type 3	606526	2q37
AP3 β-subunit	Hermansky Pudliak 2	603401	5q14.1

(glycosylasparaginase (aspartylglucosaminidase)) cause uniquely accumulation of soluble oligosaccharides. Typical symptoms are mild mental retardation, recurrent infections, dysostis multiplex and hearing loss. Often these patients survive into adulthood.[38,42]

Defects in Glycolipid Degradation

This group includes defects in:

a. Degradation of G_{M1} ganglioside
b. Degradation of sulfatide
c. Degradation of globotriaosylceramide

Gangliosides consist of a lipid moiety linked to a number of oligosaccharide structures differing in glycosidic linkage position, sugar configuration, neutral sugar and sialic acid content. The main gangliosides of the central nervous system (CNS) belong to the ganglio series, that is characterised by the tetrasaccharide Gal(β1–3)GalNAc(β1–4)Gal(β1–4)Glc to which residues of sialic acids are linked[43] (Fig. 3). The sulfatides are galactosylceramides to which

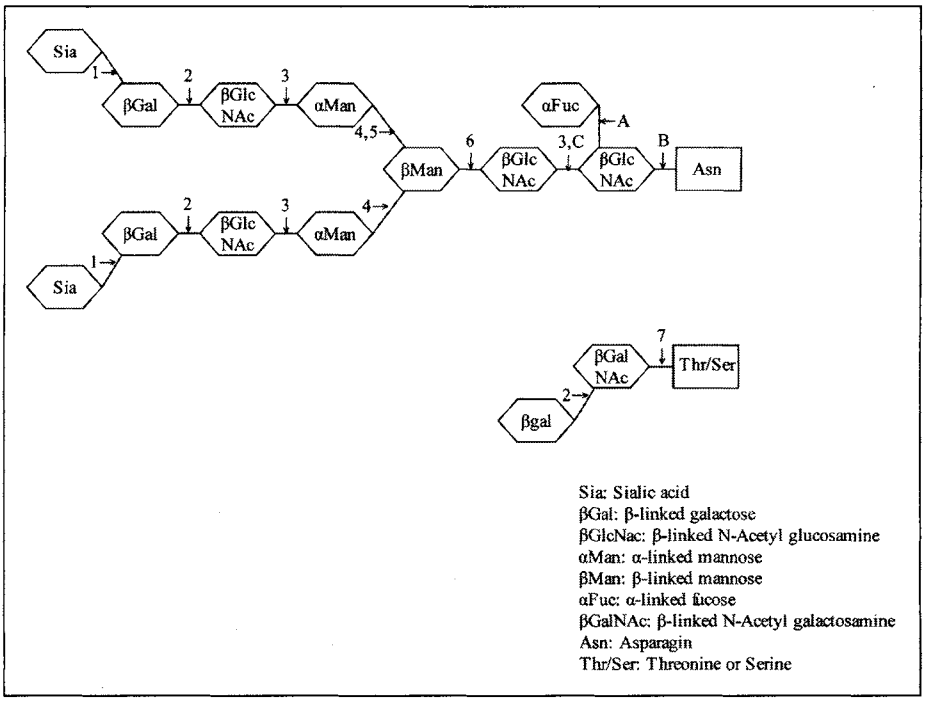

Figure 2. Degradation of N-linked and O-linked glycans. Two sets of reactions (1-7 and A-C) act in sequence from opposite ends in these degradation pathways. The reactions are numbered according to their order in this stepwise process. Reaction 1-6: breakdown from the nonreducing end by the exoglycosidases; (1) α-sialidase, requiring cathepsin A (2) β-galactosidase, requiring cathepsin A, (3) β-hexosaminidase, (4) α-mannosidase, (5) α (1-6) mannosidase and (6) β-mannosidase. (7) α-N-acetylgalactosaminidase. Reaction A-C: Sequential reactions from the reducing end by (A) α-fucosidase, (B) glycosylasparaginase and (C) chitobiase.

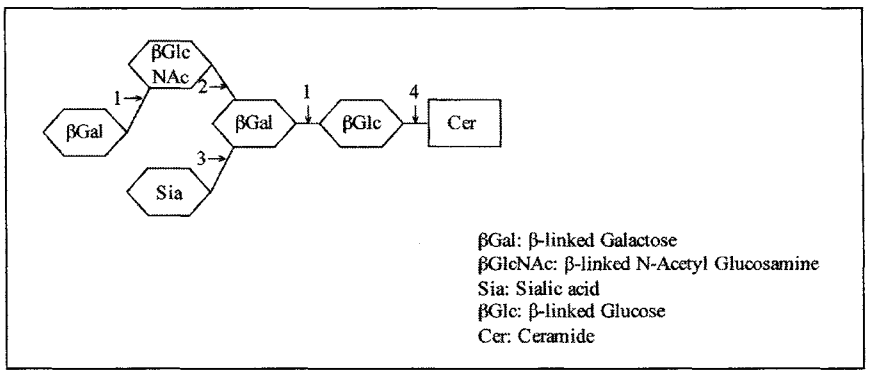

Figure 3. Degradation of G_{M1} ganglioside. (1) β-galactosidase, (2) β-hexosaminidase A, requiring GM2 activator protein (3) sialidase (4) glucocerebrosidase, requiring saposin C.

sulphate is O-linked in 3-position.[44] Galactosylceramide and sulfatide constitute a major part of the myelin layer in the CNS.[45] As gangliosides and sulfatides serve important functions in brain membranes, the lack of enzymes involved in their degradation will change the lipid

composition in brain and thus cause severe brain diseases. These diseases also exist as juvenile and adult forms associated with low residual enzyme activities. An exception is the hydrolysis of globotriaosylceramide by alpha-galactosidase A. Since this glycolipid is mainly represented in peripheral tissues, the lack of alpha-galactosidase A causes only mild symptoms without brain involvement.[46]

Degradation of G_{M1} Ganglioside

The degradation pathway of the G_{M1} ganglioside is illustrated in Figure 3 and the respective diseases caused by the lack of hydrolase 1 (beta-galactosidase), hydrolase 2 (beta-hexosaminidase) and hydrolase 4 (beta-glucocerebrosidase) are outlined in the table. The complete lack of these hydrolases results in early onset of symptoms usually about 4 months of age, severe neurological problems and death before the age of 4 years.[12,13,15] The chronic (mild) forms of the defects can be quite complex showing a variety of clinical symptoms. The mild forms of beta-galactosidase deficiency (MPS IVB) and glucocerebrosidase deficiency (Gaucher disease type I) exhibit low residual activities and do not cause neurological deterioration. MPS IVB may be caused by the inability to degrade keratan sulfate. Gaucher disease type I results in engorged macrophages causing enlargement and dysfunction of the liver and spleen and damage to bone. The progression of the disease occurs slowly over decades.[13] Gaucher disease type I was the first lysosomal storage disease that was successfully treated with enzyme replacement.[22]

Degradation of Sulfatide

The inability to degrade sulfatides will cause accumulation of storage material in the brain and severe neurological symptoms, particularly by demyelination. Usually the disease begins at 3 months of age and soon progresses to severe mental and motor deterioration causing death before age of 2 years.[44,47] Milder forms of the diseases causing a spectrum of clinical severities of brain disorders have been reported. The degradation of sulfatide occurs in two steps. In the first step the sulfate group is cleaved by 3-O-sulfogalactosyl cerebroside sulfatase (arylsulfatase A). The lack of this enzyme as well as the activator protein saposin B and Formyl-Glycin generating enzyme (FGE) which is required for the posttranslational modification of a cysteine in arylsulfatase A and other lysosomal sulfatases, cause symptoms typical for metachromatic leukodystrophy.[44,48] In the second step the β-galactosyl group is cleaved from galactosylceramide. The lack of this cleavage causes Krabbe disease (globoid cell leukodystrophy).[47]

Degradation of Globotriaosylceramide

The globotriasylceramide is a glycolipid that is predominantly found in the vascular endothelium and not in the nervous tissue. Thus the defective degradation of this molecule does not affect the brain function to any large degree. Fabry disease is caused by the lack of lysosomal α-galactosidase. As the gene is X-linked the disease primarily affects males. Clinical manifestations appear in childhood or adolescense with pain in the extremities, angiokeratoma, hypohidrosis and retarded growth.[46] The deposition of glycolipids occurs predominantly in lysosomes of endothelial, and smooth muscle cells of blood vessels. With increasing age severe renal impairment leads to renal failure, which contributes to the death. Enzyme replacement therapy is available for Fabry disease.[23]

Defects in Glycosaminoglycan Degradation (Mucopolysaccharidoses)

Defect in the degradation of glycosaminoglycans characterises the disease group mucopolysaccharidosis (MPS) (Table 1). Figure 4 illustrates the enzymes required in the degradation of heparan sulphate. The diseases in the MPS group share in a variable degree the clinical phenotypes. These include organomegaly, dysostis multiplex, decreased growth, recurrent infections and a chronic, progressive course of the disease.[49] Most of the diseases do not affect the nervous system, and the disorders have thus been considered as potentially

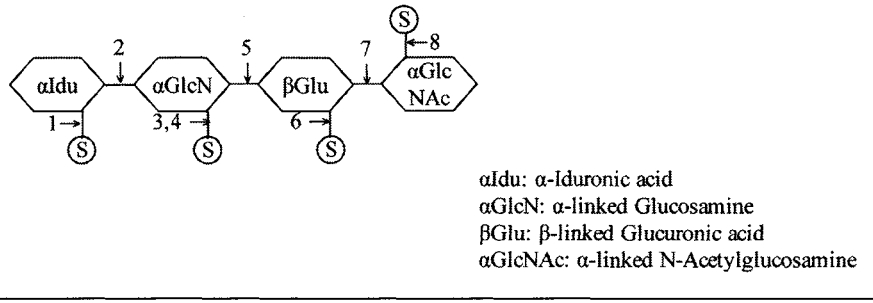

Figure 4. Degradation of heparan sulphate Enzymes participating in this degradation pathway are (1) Iduronate sulfatase (2) α-Idorunidase (3) Heparan sulfatase (4) Acetyl-CoA transferase (5) N-acetyl glucosaminidase (6) Glucuronate sulfatase (7) β-glucuronidase (8) N-acetyl glucosamine 6-sulfatase.

amenable to enzyme replacement therapy. Such therapy is available for MPS I,[24] while clinical trials are ongoing in MPS II[27] and MPS VI.[28] As the degradation of different types of glycosaminoglycans may include similar glycosidases and sulfatases, the types of degradation products due to a single enzyme defect may originate from different types of partially degraded glycosaminoglycans.

As shown in Figure 4, the first two step in the heparan sulfate degradation is catalysed by hydrolase 1 (iduronate sulfatase) and hydrolase 2 (alpha-iduronidase) which is required for the desulfatation of 2-sulfated iduronic acid residues and iduronic acids respectively, in dermatan sulfate and heparan sulfate. The lack of these enzyme activities causes Hunter syndrome (MPS II) and Hurler syndrome (MPS I) respectively.[49] The gene encoding iduronate sulfatase is localised to chr. X and MPS I is thus restricted to males. The severe form of MPS I and II usually occurs between 2 and 4 years of age with progressive neurological and somatic involvement and death between 10 and 15 years. The mild forms are characterised by preservation of intelligence and survival into adulthood. The clinical variation is probably caused by residual enzyme activities.[49] The deficiencies of hydrolase 3 (heparan N-sulfatase), enzyme 4 (Acetyl-CoA acetyltransferase), hydrolase 5 (alpha-N-acetylglucosaminidase) and hydrolase 8 (N-acetylglucosamine 6-sulfatase) cause Sanfilippo syndromes A-D (MPS IIIa-d),[49] (Table 1). The syndromes are characterised by degeneration of the central nervous system (CNS) and mild somatic disease. The onset is usually between 2 and 4 years of age and severe CNS disorder is apparent by 6 to 10 years of age followed by early death.

Hydrolase 7 (β-glucuronidase) removes the glucuronic acid residues present in dermatan sulfate, chondroitin sulfate, dermatan sulfate and hyaluronan. The disease is characterised by dysostis multiplex, dysmorphic features, hepatosplenomegaly with a range of clinical severities. The infantile form resembles Hurler syndrome (MPS I). The mental retardation is modest. Hydrolase 6 (glucuronate-2-sulphatase) has not yet been associated with a lysosomal disease.[49] Two lysosomal storage disorders in this group do not affect a hydrolase involved in the heparan sulfate degradation. These are N-acetylgalactosamine 4-sulfatase deficiency that cause Maroteaux-Lamy syndrome (MPS VI) and galactose 6-sulfatase deficiency that cause Morquio syndrome type A (MPS IVA), resulting from defects in dermatan and keratan sulfate degradation respectively. These diseases are characterised by short trunk dwarfism, skeletal dysplasia and mostly preservation of intelligense with an onset of symptoms at 2-4 years of age.[49] The lack of lysosomal hyaluronidase causes the very rare disease MPS IX. The clinical symptoms of a single patient showed mild symptoms, including notable periarticular soft tissue masses, mild short stature, an absence of neurological or visceral involvement, and histological and ultrastructural evidence of a lysosomal storage disease.[73]

Defects in Glycogen Degradation

The defective degradation of glycogen in the lysosomes is caused by the lack of a single enzyme, lysosomal acid alpha-glucosidase resulting in glycogen storage disease type II (Pompe disease).[14] The classic infantile form of the disease causes cardiomegaly, hypotonia, hepatomegaly and death before 2 years of age due to cardiorespiratory failure. There is an extreme variation of the clinical severities usually with skeletal muscle and cardiac involvement and a slower progressive course as compared to the infantile form. The milder forms appear to be associated with low residual activities. Clinical trials on enzyme replacement therapy are ongoing.[26]

Defects in Lipid Degradation

The defects in lipid degradation involve the two steps degradation of sphingomyelin to sphingosine and the ester hydrolysis of triglycerides and cholesteryl esters. The first step in the sphingomyelin degradation is a phosphodiester cleavage into ceramide and phosphocholine by acid sphingomyelinase. The lack of this enzyme results in Niemann Pick disease types A and B.[50] Niemann Pick disease type A is an infantile form resulting in growth failure, hepatosplenomegaly and severe brain damage and leads to death before 2 years of age. Niemann pick disease type B is associated with low residual activity of acid sphingomyelinase and is phenotypically variable with typical symptoms as hepatosplenomegaly and pulmonary diffusion. Most of these patients have little neurological involvement and survival into adulthood. The second step in the sphingomyelin breakdown is the deamidation of ceramide into sphingosine and free fatty acid by acid ceramidase. The lack of this enzyme causes Farber disease.[51] The classic infantile disease is characterised by painful swelling of joints, subcutaneous nodules and progressive hoarseness. In many cases there is a severe impairment of psychomotor development. Death usually occurs within 3 years of age. In the intermediate and mild forms of the disease the severities of the clinical symptoms vary considerably with life span ranging up to adulthood. The degrees of severities appear to correlate with the rate of sphingomyelin-derived ceramide degradation.[51]

The ester hydrolysis of triglycerides and cholesteryl esters is catalysed by acid lipase. Wolman disease is the infantile form of acid lipase deficiency causing hepatosplenomegaly, various gastrointestinal symptoms, adrenal calcification, failure to thrive. Death occurs before 1 year of age.[52] Cholesteryl ester storage disease is a milder form caused by a low residual acid lipase activity. Typical clinical symptoms are atherosclerosis and hepatomegaly without mental retardation. In some patients hepatomegaly in adulthood may be the only symptom.

Defects in Protein Degradation

Diseases caused by the deficiency of lysosomal proteases are rare among the lysosomal storage disorders. Three disorders caused by the lack of cathepsin K, tripeptidyl-peptidase and palmitoyl-protein thioesterase are so far the only proteinase deficiencies reported. The lack of cathepsin K activity causes pycnodysostosis and the osteclasts in such patients display reduced capacity to degrade bone matrix during bone growth and remodelling. The disease is primarily a bone disease and common symptoms include short stature with a number of skeletal anomalies with typical dysmorphic features.[53] Life span and intelligence are normal.

Lysosomal accumulation of autofluorescent, ceroid lipopigment material in various tissues and organs of which only brain tissue shows severe dysfunction and cell death are common features of the neuronal ceroid lipofuscinoses (NCLs). The deficiencies of palmitoyl-protein thioesterase (CLN1) and tripeptidyl-peptidase I (CLN2) result in the infantile (INCL) and late infantile (LINCL) forms of neuronal ceroid lipofuscinoses respectively.[54] The symptoms are progressive neurogeneration, seizures, spacicity, dementia and blindness. The symptoms appear at about 1 year of age and death occurs around the age of 8 for INCL whereas the symptoms appear later in LINCL.[54] Palmitoyl-protein thioesterase-deficiency (INCL) is prevalent in Finland with a carrier frequency of 1/50. The cause of the disease is not known, but may be related to a lysosomal accumulation of thioester-linked peptides in neurons. The physiological function

of tripeptidyl peptidase is also not known. Functions in the degradation of neuropeptides[55] and a role in antigen processing[56] have been proposed.

Defects in Lysosomal Transporters

After lysosomal hydrolyses of macromolecules in the lysosomes the building blocks as monosaccharides and amino acids are transported through the lysosomal membrane into cytosol. Mutations in the sialic acid transporter (sialin) cause sialic acid storage disease.[57] The infantile form of the disease (ISSD) is characterised by failure to thrive, hepatosplenomegaly, severe mental and motor retardation and dysostis multiplex. The children usually die before the age of 1 year. The juvenile/adult form of the disease is called Salla disease due to its prevalence in the Salla region of Finland. The children are born healthy, but develop psychomotoric delay and ataxia in infancy. Intelligence is moderately to severely reduced and they usually survive into adulthood.

Defiency of the cystine transporter (cystinosin) results in the storage disorder cystinoses. The affected children are usually born healthy and develop signs of kidney disease before age of 1 year.[58] Symptoms include dehydration, acidosis, vomiting, failure to grow. The renal glomerular damage progresses with age, requiring dialysis or transplantation at 6 to 12 years of age. Oral cysteamine therapy has proven to be efficient in the systemic depletion of cystine, and patients receiving this drug before the age of 2 years display a delayed clinical onset of the disease.

Defects in Trafficking

Deficiencies in trafficking have recently been recognised to cause several lysosomal disorders. The deficient proteins may not be directly linked to a lysosomal location, but may be present in the trafficking route of lysosomal proteins from endoplasmic reticulum (ER) to the lysosomes. Thus, both cytosolic proteins as well as ER/Golgi/endosome/lysosome localised proteins involved in trafficking may cause lysosomal storage disorders.

Given the large number of proteins involved in trafficking one may expect the discovery of new protein deficiencies of this group in the near future.

The mucolipidosis diseases are due to the simultaneous lysosomal storage of lipids together with water-soluble substances. The classical trafficking defects are the mucolipidosis type II and III (I-cell disease). These diseases are caused by the defective activity of UDP-N-acetylglucosamine-phosphotransferase, a cis-Golgi localised enzyme that recognises and attaches GlcNAc-1-P onto terminal mannose-residues on N-glycans in lysosomal hydrolases.[59] Defects in this transferase cause mislocalisation of lysosomal hydrolases into the extracellular milieu. The transferase is composed of two gene products, the α/β-subunit and the gamma-subunit. Mutations in the γ-subunit appear to be the major cause of mucolipidosis type III. This is a mild disorder with onset of symptoms about 2 to 4 years of age and a slow progression of symptoms with survival into adulthood.[60] The symptoms share many features with the mild form of MPS I. Mucolipidosis type II is a more severe disease with death before the age of 8 years, but so far no mutations causing this disease has been reported.

Mucolipidosis type IV (MLIV) is characterized by psychomotor retardation and ophthalmological abnormalities. Severely affected as well as milder patients have been described. Over 80% of the MLIV patients are Ashkenazi Jews; the estimated heterozygote frequency in this population is 1/100.[61] A broad spectrum of storage material stems from an abnormal endocytosis process in cells from MLIV patients. The missing protein, mucolipin-1 is a cation channel that seems to be involved in the acidification and normal endosomal function.[4]

Deficiency of LAMP-2 (Danon disease) belongs to the hereditary myopathies characterized by the development of autophagic vacuoles.[62] LAMP-2 deficiency is a rare X-linked disorder.[63] It is characterized clinically by cardiomyopathy, myopathy and variable mental retardation. The pathological hallmark of the disease is intracytoplasmic vacuoles containing autophagic material and glycogen in skeletal and cardiac muscle cells. The role of LAMP 2 is not yet unravelled, but it appears to be linked to the trafficking of lysosomal hydrolases.[64]

The defect in trafficking of cholesterol causing lysosomal/endosomal accumulation of unesterified cholesterol results in Niemann Pick disease type C (NP-C). This accumulation is particularly pronounced in liver and spleen but more deleteriously in brain, where it correlates with severe neuronal dysfunction.[65] About 95% of the patients have mutations in the NPC1-gene, and the remainder in the NPC2-gene.[66] Although the functions of these gene products are not known, it is believed that NPC1 is involved in regulation of the membrane trafficking in the endosomal/lysosomal system. The clinical manifestations are heterogeneous. Most patients have progressive neurological disease and hepatosplenomegaly. The symptoms appear most often in childhood and death occurs in the teenage years or early adulthood. The adult forms of NP-C cause psychiatric illness and dementia.

The neuronal ceroid lipofuscinoses (NCL) are a group of diseases that are characterized by progressive accumulation of autofluorescing waxy lipopigments (ceroid-lipofuscin) within the brain, accumulation of hydrophobic proteins in lysosomes, progressive neuroretinal symptomatology and cerebral atrophy. NCLs are caused by at least 8 mutant genes (CLN1-CLN8).[67] Several lines of evidence have suggested that the CLN-gene products have roles in the protein trafficking in the endosomal/lysosomal system. Whereas the soluble lysosomal hydrolases CLN1 and CLN2 appears to be involved in lysosomal/endosomal proteolysis, CLN3, CLN6 and CLN8 are membrane proteins that may be involved in vesicular trafficking. CLN3-deficiency causing juvenile neuronal ceroid lipofuscinosis (Batten disease) is a form of NCL that is characterized by onset of neuroretinal symptoms between 4 and 10 years.[67] It is the most common type of NCL in the United States and Europe. CLN3 has a proposed orthologue in Drosophilia, BTN1 that appears to be linked to the regulation of endocytosis.[68] CLN6 and CLN8 are both transmembrane protein residing in the endoplasmic reticulum.[69,70] CLN8-deficiency causes progressive epilepsy with mental retardation (Northern epilepsy). It is characterized by the onset of generalized seizures at between five and ten years of age, with progressive deterioration of mental development thereafter.[67] The symptoms of CLN6-deficiency are similar. The functions of CLN6 and 8 remain to be found. Based on sequence homology analysis CLN8 has been suggested to be involved in lipid synthesis in the endoplasmic reticulum,[70] but the relation to lysosomal dysfunction remains obscure. Possibly these proteins are involved in the ER to Golgi transport of lysosomal proteins.

The concept of lysosomes not only having an endpoint function in the degradation, but also function in the regulated secretion in immune cells and melanocytes, has resulted in the finding of a number of lysosomal diseases where the trafficking of the lysosomes to the plasma membrane is deficient.[71] A number of human autosomal diseases give rise to both pigmentation and immune dysfunction, which is the hallmark for this group of lysosomal storage diseases. The first gene to be mapped was from patients affected with Chediak-Higashi syndrome. The patients are characterised by hypopigmentartion and enlarged lysosomes in all cell types. The defective gene LYST (lysosomal trafficking regulator) is a cytosolic protein that plays a role in regulating membrane fusion. Patients affected with Griscelli syndrome also exhibit hypopigmentation but the lysosomes are normal in size. The defective genes are RAB27A (type 2) and MYOV (type 1) and melanophilin (type 3) which are involved in the fusion of melanosomes with the plasma membrane. Patients affected with Chediak-Higashi and Griscelli syndromes have severe neurological problems due to infiltration of activated T-cells and macrophages in the brain. The disorders known as Hermansky-Pudlak syndrome (HPS) are a group of genetic diseases resulting from abnormal formation of intracellular vesicles. The respective proteins contribute to the formation of organella such as melanosomes and lysosomes. The syndrome has evolved into a group of genetically distinct disorders characterized by oculocutaneous albinism, a storage pool deficiency, and impaired formation or trafficking of intracellular vesicles.[71,72] There are now seven disorders in this group, but taking into account that there are 16 mouse models of this syndrome, it is likely that new genetic types of Hermansky-Pudlak syndrome will appear.[74] The best characterised protein defect is the one that causes Hermansky-Pudliak type 2 (HPS-2).[75] This disorder is caused by mutations in ADTB3A,

which codes for the cytosolic protein beta3A subunit of the adaptor protein-3 complex, AP3. This coat protein complex has been localized to the clathrin coat of trans Golgi network as well as to a peripheral endosomal compartment. AP3 is known to play a role assisting in vesicle formation from the trans-Golgi network or late endosomes.

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CHAPTER 7

Lysosomal Transporters and Associated Diseases

Frans W. Verheijen* and Grazia M.S. Mancini

Abstract

The lysosomal organelle system is the main vacuolar site for the turnover of endogenous and exogenous macromolecules. The lysosomal lumen contains a large number of predominantly hydrolytic enzymes for these catabolic steps with characteristic acidic pH-optima and high substrate specificity. The lysosomal membrane is formed by a typical single phospholipid bilayer which controls the passage of material into and out of lysosomes, by its permeability and ability to fuse with other (digestive) vacuoles. By the late seventies it became clear that the lysosomal membrane plays an important role in the disposal of metabolites produced by enzymatic degradation of macromolecules inside the lysosomal compartment. Initially the lysosomal membrane was considered to be only a mechanical border separating the acid lysosomal environment from the neutral surrounding cytoplasm. Since the discovery of a lysosomal cystine carrier, defective in an inherited human disease, more than 20 specific transport systems have been characterized in the lysosomal membrane.[1] Most of them function as exporters and only a few as importers. Several types of lysosomal membrane transporters can be discriminated: solute carriers, pumps and channels. Each of the lysosomal transporters has a high specificity for groups of amino acids, sugars, nucleosides, inorganic ions, and vitamins. Genetic disorders of these transporters cause a wide array of neurological and visceral diseases, ranging from developmental to degenerative disorders. Until recently, all knowledge about lysosomal transport proteins was based on the biochemical (kinetic) characteristics of transport. For biochemical and kinetic properties regarding each system, readers are referred to previous reviews.[2-4] The molecular and functional properties of the better characterized lysosomal transport systems and the related human diseases are discussed here.

The Lysosomal Membrane and Storage Diseases

Lysosomes are intracellular organelles acidified by a vacuolar-type (V-type) proton pump, which lowers the intraluminal pH to around 5. This acid environment is essential for several lysosomal functions, like enzymatic degradation, proton-coupled transport processes, receptor-ligand interactions, vesicle trafficking and sorting. In lysosomal storage diseases, undegraded macromolecules accumulate in the lysosomal compartment as a consequence of the mutation in one of the lysosomal hydrolases.[5,6] However, in a few cases the substances accumulated in the lysosome are not undegraded macromoleculas but products of hydrolytic degradation that are supposed to leave the lysosomal compartment for metabolic recycling. In the group of lysosomal storage diseases, transport disorders represent rare examples of inborn errors of metabolism caused by a defect of an intracellular membrane transport. The occurrence of such human genetic disorders provides an important tool to understand fundamental

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Table 1. Genetic disorders of lysosomal transporters: Classification

Gene/ Protein	Chromosome	Disease	# MIM	Protein Function
CTNS Cystinosin	7q13	Cystinosis	219800 219900 219750	H+-driven cystine transporter
SLC17A5 Sialin	6q14-q15	Sialic acid storage disease	604369 269920	H+-driven acid sugar transporter
CLN3 CLN3 protein	16p12.1	Batten disease	607042	Arginine transport?
NPC1 NPC1 protein	18q11	Niemann-Pick type C1	257220	H+-driven acriflavin and fatty acid pump
SLC36A1 LYAAT-1	5q33.1	-	606561	Small neutral amino acids
TCIRG1 A3subunit	11q13.4-q13.5	Infantile malignant osteopetrosis	605239	A3 subunit vacuolar H+-ATPase
CLCN7 Chloride channel	16p13.3	Infantile malignant osteopetrosis	602727	Chloride channel
MCOLN1 Mucolipin	19p13.3-p13.2	Mucolipidosis IV	252650	Ca++ channel?

cellular mechanisms and protein functions. Except for most enzymatic functions, it is now clear that many lysosomal functions are carried out through specialized proteins situated in the lysosomal membrane. The membrane contains:

1. The transmembrane transporters carrying solutes and degradation products across the membrane.
2. The membrane proteins involved in the acidification process: the multimeric protein complex of the electrogenic proton pump (V-type H+-ATPase) and the chloride channels shunting the generated electric current.
3. Several other heavily glycosylated integral membrane proteins (Lysosome Associated Membrane Proteins, LAMPs) that play a role in the fusion between lysosomes and other organelles like autophagosomes. Other membrane associated (not always integral) proteins contribute both to enzymatic degradative functions (example: glucocerebrosidase) or to vesicle trafficking (example: Rab proteins).

Disease-causing mutations in genes encoding for some of these proteins have been only described in the last decade (Table 1), showing that the rapid progress in this research field is due to the achievements of the human genome project. Here we will focus on the disorders caused by mutations in the solute carriers, ion channels and proton pump. The only disorder caused by a defect of the heavily glycosylated integral membrane protein LAMP-2, Danon's disease (MIM 309060), is a topic of another chapter. Although it is a defect in organelle transport and communication, it is not a defect in a transport process across the lysosomal membrane.

Solute Carriers and Transport Defects

The lysosomal membrane contains several specific carriers for the transport of solutes across the membrane. Most of the substrates transported by the lysosomal carriers are products of enzymatic degradation of macromolecules (single amino acids, dipeptides, monosaccharides, and lipids), but also specific carriers transport vitamins, heavy metals and drugs.[7] Many carriers with selective substrate specificity function as uniporters (passive transporters) following the Michaelis-Menten kinetics of transport along the substrate concentration gradient, or cotransporters (symportes and antiporters, secondary active transporters) coupled to an ion gradient, which provides the driving force for the direction of transport. This is usually the proton gradient generated by the energy-dependent vacuolar proton pump. Although more than 20 carriers have been characterised, only eight genes are known of which seven are coupled to a human disease (Table 1).

CTNS encodes for cystinosin the transporter defective in cystinosis, a lysosomal storage disease caused by intralysosomal storage of cystine crystals. *SLC17A5* is the gene encoding for sialin, the sialic acid transporter defective in sialic acid storage disease. *CLN3* encodes for a multimembrane-spanning protein (transporter?), which is mainly localized in lysosomes in nonneuronal cells and in endosomes in neuronal cells. This protein is affected in Batten disease, a juvenile form of ceroid lipofuscinosis. *NPC1* encodes for a new type of human permeases and is mutated in Niemann-Pick type C1 patients.[8] *SLC36A1* encoding for a lysosomal transporter, LYAAT-1, of small neutral amino acids, like alanine, proline and GABA, has recently been identified as a member of the eukaryotic specific amino acids/auxin permease (AAAP) family, but is so far not coupled to a human disease.[9]

Cystinosin, *CTNS* and Cystinosis

There are three clinical forms of cystinosis: infantile, adolescent and adult cystinosis.The classical form of cystinosis (infantile or nephropatic cystinosis) occurs in 95% of the patients, with an incidence of approximately 1 in 200,000 live births. The disease is particularly common in Brittany with an incidence of 1 in 26000. The basic defect is an impaired function of the lysosomal cystine transporter, leading to lysosomal accumulation of free cystine. The diagnosis is based on measurement of cystine levels in polymorphonuclear leukocytes and cultured fibroblasts, also useful to distinguish cystinosis variants. Cystine crystals deposited in the kidney cause a generalized defect in proximal tubule function, leading to impaired reabsorption of small molecules (Fanconi renal tubulopathy). Patients develop renal Fanconi syndrome in the first year of life. Other clinical features are failure to thrive, delayed growth, progressive renal failure within the first decade, and a variety of other complications, including photophobia and corneal erosions due to cystine crystal formation within the eye and dysfunction of multiple endocrine glands.[6] Milder variants of the classical disease include an intermediate form with late-onset renal disease (adolescent cystinosis), and a benign form with corneal involvement but no renal impairment (adult cystinosis). The different clinical forms of cystinosis are allelic and due to mutations within the same gene. The causative gene, *CTNS*, encodes a transmembrane protein, cystinosin, which was recently identified as a H^+-driven cystine transporter using an in vitro transport assay.[10,11] Mutation analysis of *CTNS*, correlated to a cystinosis clinical severity score, suggests a good genotype/phenotype correlation.

Cystinosin is a 367 amino acid protein with seven predicted transmembrane domains. Most established solute transport proteins possess 6 or 12 transmembrane domains. The protein appears to have an uncleavable N-terminal signal sequence followed by seven glycosylation sites and a hydrophobic GYDQL sequence—a tyrosine-based lysosomal sorting signal close to the C-terminus—both features common to the two integral lysosomal-associated membrane proteins (LAMPs).[12] Besides this hydrophobic sequence, also a novel lysosomal targeting sequence, acting as a conformational motif in the third cytosolic loop has been identified. The cystine transport defect in cystinosis causes an accumulation of cystine in a host of cells, which destroys various tissues at different rates. The treatment of cystinosis is based on prevention

and treatment of renal and endocrine failure and on depletion of cystine by cysteamine. Oral administration of cysteamine bitartrate capsules (Cystagon^{R}) and cysteamine eye drops have proven their effectiveness enhancing growth, retarding renal glomerular deterioration and the need for thyroxin supplementation and dissolve the corneal crystals responsible for the photophobia. Cysteamine is able to enter lysosomes, and to react with free cystine to form free cysteine and the mixed disulphide of cysteamine and cysteine. The latter compound, an analogue of lysine, exits lysosomes via the lysosomal membrane carrier for cationic amino acids (system c), which is intact in cystinosis. After the characterisation of the cysteamine transporter it became clear how cysteamine enters the lysosome.[13]

A mouse model of cystinosis was recently generated and Ctns(-/-) mice accumulate cystine in all tissues. A high level of cystine accumulates in the kidney, but these mice do not present with proximal tubulopathy or renal dysfunction. The Ctns(-/-) mouse model may provide clues to the cause of the Fanconi syndrome associated with cystinosis, the origin of which remains poorly understood.[14]

Sialin, *SLC17A5* and Sialic Acid Storage Disease

Sialic acid storage disease is characterised by the accumulation of the acid monosaccharide sialic acid in the lysosomal compartment.[6] The diagnosis is based on the demonstration of abnormal excretion of free (nonoligosaccharide bound) sialic acid in urine and accumulation in cultured fibroblasts, and on microscopical evidence of increased and swollen lysosomes, filled with light fibrillogranular material. Sialic acid storage disease must be discriminated from other forms of sialic acid storage, namely sialidosis, a defect of the lysosomal enzyme sialidase (MIM 256550) and nonlysosomal sialuria (MIM 269921).

There are different clinical forms of this autosomal recessive disorder. The adult form, also called Salla disease (SD) or Finnish sialuria (MIM 604369), the infantile sialic acid storage disease (ISSD, MIM 269920) and an intermediate form (severe Salla disease).[15] Nystagmus, hypotonia and ataxia by the end of the first year are early signs of SD. Deep mental retardation, dementia, loss of deambulation and of acquired speech characterise the further course, which spans over several decades. A peculiar diffuse developmental defect of the cerebral and cerebellar white matter (hypomyelination and dysmyelination) is that observed at brain MRI scans. While most of the patients with SD have Finnish origin, the disease has been sporadically described in Sweden and the Netherlands (G.M.S. Mancini, personal observation). Patients with the intermediate phenotype show, besides moderate signs of neurodegeneration, marked growth retardation and delayed puberty associated with endocrine failure.[16] ISSD shares identical pathological and biochemical abnormalities with Salla disease. However the course is severe. Early psychomotor retardation, failure to thrive, hepato-splenomegaly, cardiomyopathy, nephrosis and multiple dysostosis are prominent features. Foetal hydrops and hydrocephalus can complicate the picture. ISSD has been described in about 30 patients world-wide.

The locus for SD and ISSD was mapped on chromosome 6q14-q15 by linkage analysis. DNA analysis of the *SLC17A5* gene confirmed that SD and ISSD are allelic disorders. Mutation analysis in SD showed a "founder" missense mutation (115 C>T, R39C) in exon 2 in almost all Finnish patients. This mutation seems to be associated with a milder course also in patients from other European countries either at the homozygous or heterozygous state. 10 different ISSD mutations have been found throughout the gene in 15 unrelated patients, showing that no particular mutation is associated with this phenotype (Verheijen et al personal observation).[17,18] However, a (802-816)15 bp deletion in exon 6 has been found in 9 unrelated patients from Canadian, English, French, Italian and Polish origin (Mancini and Verheijen, personal observations).[15] *SLC17A5* encodes for sialin, a carrier with 12 transmembrane domains belonging to the ACS family of anion/cation symporters. Biochemical transport studies before cloning of the gene had previously demonstrated that sialic and glucuronic acid transport in the lysosome is mediated by a secondary active carrier driven by the proton gradient generated by the vacuolar proton pump. Fibroblasts from patients with SD and ISSD showed impaired

transport of both sugars across the lysosomal membrane.[2,7] The ACS family in humans includes Na^+/phosphate cotransporters and two brain-specific carriers BNPI (VGLUT1, *SLC17A7*, MIM 605208) and DNPI (VGLUT2, *SLC17A6*) highly homologous to *SLC17A5* (37 % identical and 61% similar amino acids). BNPI and DNPI are proton-driven transporters of synaptic vesicles for glutamate, the main excitatory neurotransmitter of CNS.[19,20] While it remains unexplained how a transport defect for a monosaccharide leads to a brain developmental defect and to neurodegeneration, it is worth to mention that this transporter shows a wide substrate specificity, apparently extended also to non sugar anions.[21]

In the CNS, sialin is predominantly expressed in neurons, especially in the proliferative zone of the prospective neocortex and the hippocampus in developing brain. In nonneuronal cells and primary glial cell cultures, mouse sialin is localized into lysosomes but interestingly, in primary neuronal cultures sialin is not targeted into lysosomes but rather revealed a punctate staining along the neuronal processes and is also seen in the plasma membrane. A nonlysosomal localization of sialin in neurons implies a possible role for sialin in the secretory processes of neuronal cells.[22]

CLN3 and Batten Disease (Juvenile Ceroid Lipofuscinosis)

Batten disease is one of the most common progressive neurodegenerative disorders of childhood, resulting from autosomal recessive inheritance of mutations in the *CLN3* gene (see also Chapter 8). Pathologically, Batten disease is characterized by lysosomal storage of autofluorescent material in all tissue types. Although characterized by seizures, mental retardation, and loss of motor skills, the first presenting symptom of Batten disease is vision loss.[23] The endosomal/lysosomal transmembrane protein CLN3 is mutated in Batten disease (juvenile neuronal ceroid lipofuscinosis, JNCL). Protein secondary structure prediction programs suggest that the CLN3 protein has five to seven membrane-spanning domains. However, the molecular mechanism of JNCL pathogenesis and the exact function of the CLN3 protein have remained unclear. The *BTN1* gene, the yeast orthologue of human *CLN3* gene (not to be confused with the yeast *CLN3*, a G1 cyclin!) encodes an integral lysosomal membrane protein with several transmembrane domains, important for lysosomal acidification and cellular pH homeostasis.[24] Deletion of yeast *BTN1* (btn1-delta), an ortholog of the human Batten disease gene CLN3, results in a decrease in vacuolar pH during early growth. The altered vacuolar pH in btn1-delta strains underlies a lack of arginine transport into the vacuole, which results in a depletion of endogenous vacuolar arginine levels. This arginine transport defect in btn1-delta is complemented by expression of either *BTN1* or the human CLN3 gene and strongly suggests a function for transport of, or regulation of the transport of, basic amino acids into the vacuole or lysosome for yeast Btn1p, and human CLN3 protein, respectively.[25] Novel interactions between the microtubule-binding Hook1 and the large family of Rab GTPases suggest a link between CLN3 function, microtubule cytoskeleton and endocytic membrane trafficking.[26] Whether Batten disease, caused by a *CLN3* mutation (MIM 204200), is a lysosomal transport defect needs to be demonstrated.

NPC1 and Niemann-Pick Disease

Niemann-Pick disease type C is a recessive neurodegenerative disorder of variable onset, characterised by lysosomal accumulation of low-density lipoprotein (LDL)-derived cholesterol and sphingomyelin.[27] The clinical signs include cholestatic jaundice and severe hepatic dysfunction, mostly in the infantile onset, supranuclear vertical gaze palsy, ataxia, extrapyramidal deficits, marked spasticity and dementia. The recycling of cholesterol after receptor-mediated endocytosis is defective (see also Chapter 9), with consequent intralysosomal cholesterol storage and insufficient recycling. The biochemical diagnosis is based on the demonstration of vesicle-bound intracellular cholesterol accumulation by filipin staining of cultured fibroblasts. The disease is genetically heterogeneous and mutations in two genes, *NPC1* on chromosome 18q11 (MIM 257220) and *HE1* (also known as *NPC2*) on chromosome 14q24.3 (MIM

601015), have been found. The *NPC1* gene encodes for a proton-driven transporter, with 13 transmembrane domains, highly homologous to bacterial RND (resistance-nodulation-division family). This transporter is involved in extracellular pumping of acriflavine and fatty acids and is homologous to Patched, a human morphogen receptor, mutated in genetic tumour syndromes like Gorlin syndrome (MIM 109400). Its relation to the defect in cholesterol recycling needs further investigation, but, noteworthy, also Patched, as a ligand for the Sonic Hedgehog, is involved in cholesterol metabolism. The *HE1* (*NCP2*) gene encodes for a soluble lysosomal protein bearing the mannose-6-phosphate marker. Its porcine homologue is known to bind specifically cholesterol, but its function in intracellular cholesterol recycling is still obscure.[28] No clinical differences have been reported between the two genetic forms of the disease.

SLC36A1 Encoding LYAAT-1, a Transporter for Small Neutral Amino Acids

LYAAT-1 (also called PAT1) is a lysosomal transporter that actively exports neutral amino acids from lysosomes by chemiosmotic coupling to the H^+-ATPase of these organelles.[29] Homology searching in eukaryotic genomes suggests that LYAAT-1 defines a subgroup of lysosomal transporters in the amino acid/auxin permease family.[9] Light and confocal microscopy demonstrated that LYAAT-1 and the lysosomal marker cathepsin D colocalized throughout the brain and electron microscopy showed that LYAAT-1-IR was associated with lysosomal membranes.[30] In addition, LYAAT-1-IR was also found associated with other membranes belonging to the Golgi apparatus and lateral saccules and less frequently with multivesicular bodies, endoplasmic reticulum, and occasionally with the plasma membrane. The localization of LYAAT-1 at the lysosomal membrane is consistent with the view that it mediates amino acid efflux from lysosomes. This transporter is not yet coupled to a genetic disease.

MCOLN1, Mucolipin and Mucolipidosis Type IV

Mucolipidosis IV is an autosomal recessive neurogenetic disorder characterized by developmental abnormalities of the brain and impaired neurological, ophthalmologic and gastric function. Large vacuoles accumulate in various types of cells in MLIV patients. However, the pathophysiology of the disease at the cellular level is still unknown. Transport from late endosomes to lysosomes results in the formation of an endosome-lysosome hybrid organelle from which late endosomes and lysosomes must be reformed. Mucolipin shows sequence homology and topological similarities with polycystin-2 and other transient receptor potential (Trp) channels. Recent studies indicate that the transient receptor potential (TRP)-related channel mucolipin-1 and its Caenorhabditis elegans orthologue CUP-5 might control the process of lysosome reformation by regulating calcium flux.[31]

TCIRG-1 and *CICN7*, A3 Subunit ATPase and Chloride Channel 7, Lysosomal Acidification Disorder, Infantile Malignant Osteopetrosis (Albers-Schonberg Disease)

Infantile malignant autosomal recessive osteopetrosis, the most severe form of the heterogeneous group of osteopetroses, is a rare genetically heterogeneous disease (MIM 259700) caused by the inability of osteoclasts to resorb and remodel bone, resulting in generalized osteosclerosis and obliteration of marrow spaces and cranial foramina. The classical clinical features are pathological fractures, visual impairment, and bone marrow failure. Two human genes have been described as the cause of this form of osteopetrosis: the T-cell immune-regulator-1 (*TCIRG1*) gene, which is mutated in >50% of the patients, and the chloride channel 7 (*ClCN7*) gene, which accounts for approximately 10% of cases.[32] The clinical signs are the result of a progressive osteosclerosis, which causes destruction of the bone marrow with pancytopenia, secondary hepatosplenomegaly, visual and hearing loss and hydrocephalus, partially due to cranial nerve compression and otosclerosis and partially due to primary nerve degeneration.

Hypocalcemia, hyperphosphatemia and elevated alkaline phosphatase are sometimes associated with tetany. The disease is also called "marble bone" disease, because of the radiological appearance of the affected skeletal segments, with increased trabecular bone density, calcified cartilage and bone-within-bone appearance. The laboratory diagnosis is based on the demonstration of intralysosomal cholesterol storage by filipin staining. Therapy consists of bone marrow transplantation. The disease is genetically heterogeneous. Mutations in two different genes have been observed: *TCIRG1* and *CLCN7*.[33]

The *TCIRG1* gene (MIM 605239) encodes for the a3 subunit of the human H^+-ATPase, the vacuolar proton pump. The H^+-ATPase protein complex acidifies various intracellular organelles, like lysosomes and endosomes. It comprises 13 different subunits and it functions as an electrogenic, strictly energy-dependent process. The subunit a3 was originally thought to be specific for osteoclasts, but later was found in other cell types. It is induced on osteoclast differentiation and is expressed in the ruffle border, the membrane structure responsible for the bone resorption. The ruffle border is a kind of "extracellular lysosome" responsible for local extracellular acidification. It contains the H^+-ATPase and is the place of extracellular secretion of several lysosomal hydrolases. A mutation in the a3 subunit leads to defective extracellular acidification at the ruffle border that impairs bone resorption. An alternative-splicing product of the same gene encodes for TIRC7 (MIM 604592) a protein expressed exclusively in immune system tissue and essential for T cell activation.

The *CLCN7* gene (MIM 602727) encodes for ClC-7, one of the ClC chloride channel family.[34] Mice deficient for the ubiquitously expressed ClC-7 chloride channel show severe osteopetrosis and retinal degeneration. ClC-7 is a lysosomal membrane protein also highly expressed in the ruffle membrane of osteoclasts. Here it provides the conductance to shunt the electrical current created by the electrogenic proton pump. In lysosomes, like in other acid organelles, this conductance is selective for chloride. Although only a few patients with Albers-Schonberg disease have been found with mutations in the ClC-7 channel, this finding suggests that chloride channels are essential for the lysosomal homeostasis. At least two other ClC channels (ClC-3 and 6) are specifically targeted to the lysosomes. Events of neural and retinal degeneration observed in knock-out mice for ClC-3 might help to elucidate new human lysosomal diseases.[35]

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CHAPTER 8

Neuronal Ceroid-Lipofuscinoses

Jaana Tyynelä*

I wish to dedicate this chapter to Prof. Pirkko Santavuori (29.4.1933-6.2.2004), the mother of Finnish NCL research.

Abstract

Neuronal ceroid-lipofuscinoses (NCLs, also referred to as Batten's disease) are inherited lysosomal storage diseases characterized by progressive neurodegeneration and premature death. As a group, the NCLs compose the most common reason for children's progressive encephalopathy, and their incidence in the Northern Europe and US has been estimated to be approximately 1:12 000. Previously, the NCLs were classified into different forms on the basis of age of onset as well as clinical and electron microscopic findings. The presently used nomenclature is based on the genetic findings and divides the NCLs into eight forms (CLN1-CLN8). Seven of the human or ovine genes underlying NCLs have been identified during the last decade, and vigorous efforts are underway to reveal the physiological functions of the respective proteins. Despite these advances, little is understood about the pathogenetic mechanisms leading to a neuron specific manifestation and neuronal death in these diseases. This review summarises the present knowledge on NCLs and focusses on the recent biochemical and cell biological advances in the field.

Classification and Genetics

Traditionally, the NCLs were classified into four major forms, the infantile (INCL), late-infantile (LINCL), juvenile (JNCL) and adult (ANCL) NCL, based on the age of onset, clinical course, as well as neurophysiological and electron microscopic findings. In addition, a number of so-called variant forms exist, leading to a complicated nomenclature. However, advances in the genetic studies have allowed a novel, pragmatic classification, and presently the NCLs are divided into eight different forms on the basis of the underlying genetic defect: CLN1-CLN8. In addition, there are at least three rare forms of NCL, which remain without genetic assignment.[1-3] The nomenclature and main characteristics of each NCL form are summarized in Table 1.

Most human NCLs are recessively inherited, the only exception this far being a dominantly inherited adult-onset human NCL, Parry disease[4,5] (presently not designated with a CLN number). A number of mutations in each NCL gene have been identified, and they are listed in a mutation database at www.ucl.ac.uk/ncl. Profound reviews focusing on the genetic aspects of NCLs have recently been published.[1,6,7]

Presently, six human and one ovine NCL gene has been identified. Three of the identified genes encode previously characterized soluble lysosomal enzymes: *CLN1* gene encodes palmitoyl-protein thioesterase 1 (PPT1),[8] *CLN2* gene encodes tripeptidyl peptidase 1 (TPP1),[9] and a mutation in cathepsin D gene is responsible for congenital ovine NCL.[10] *CLN3*,[11] *CLN5*,[12] *CLN6*[13,14] and *CLN8*[15] genes encode novel proteins with presently unknown functions.

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Table 1. Human forms of NCL

	Eponymes, OMIM Number	Chromosome	Gene	Gene Product	EM	Storage Proteins	Disease Models	References
CLN1	InfantileNCL (INCL), Santavuori-Haltia disease OMIM 256730	1p32	*CLN1*	Palmitoyl-protein thioesterase 1 (PPT1), soluble	GRODs	SAPs	2 x mouse Drosophila Yeast	8,26,28-31,39, 45,50-62
CLN2	Classical late-infantile NCL (LINCL), Jansky-Bielschowsky disease OMIM 204500	11p15	*CLN2*	Tripeptidylpeptidase 1 (TPP1), soluble	CL	Subunit c	Mouse	9,30,32,46, 65-79
CLN3	Juvenile NCL (JNCL), Spielmeyer-Sjögren disease, Batten disease OMIM 304200	16p12	*CLN3*	CLN3 protein, membrane protein	FP, CL	Subunit c	3x mouse Nematode Yeast	11,35,36,46, 90-95,98-101, 135
CLN4	Adult NCL (ANCL), Kufs disease OMIM 204300	unknown	unknown	unknown	FP	Subunit c	–	16,17
CLN5	Finnish variant LINCL OMIM 256731	13q22	*CLN5*	CLN5 protein,	RL, FP	Subunit c	Mouse	12,33,102-104
CLN6	Variant LINCL or early juvenile NCL, Lake-Cavanagh disease OMIM 601780	15q21-23	*CLN6*	CLN6 protein, membrane protein	RL, FP	Subunit c	Nclf mouse 2x ovine	13,14,34,105
CLN7	Variant LINCL (Turkish)	unknown	unknown	unknown	RL, FP	Subunit c	–	18,21,22
CLN8	Northern Epilepsy (NE), Epilepsy with progressive mental retardation (EPMR) OMIM 600143	8p23	*CLN8, EPMR*	CLN8 protein, membrane protein	RL, FP	Subunit c	Mnd-mouse	15,37,38,107
Congenital NCL	–	unknown	unknown	unknown	GRODs	SAPs	–	19,20
Dominant ANCL	Parry disease OMIM 162350	unknown	unknown	unknown	GRODs	SAPs	–	4,5,23

Abbreviations: GRODs, granular osmiophilic deposits; CL, curvilinear profiles; FP, fingerprint profiles; RL, rectilinear profiles; subunit c, mitochondrial ATP synthase subunit c; SAPs, sphingolipid activator proteins.

Neither the chromosomal location nor the underlying gene have been identified for CLN4 (Kufs disease[16,17]), CLN7 (a variant late-infantile onset NCL found in Turkey[18]), congenital NCL,[19,20] or Parry disease[4,5] (an adult onset dominant NCL). CLN4 and CLN7 are hypothetical genes yet to be identified. Certain cases clinically classified as Turkish CLN7 were recently identified as early onset variants of CLN8,[21] showing a profoundly more severe phenotype than the Finnish CLN8 cases decribed earlier.[22] Only a few cases of congenital NCL have been reported, and the etiology of these cases remain to be studied. In contrast, Parry disease is thoroughly described, and given the clinical homogeneity as well as the distinct combination of dominant inheritance, granular ultrastructure, and normal enzyme activities of PPT1, TPP1 and cathepsin D, it is likely to be caused by a novel NCL gene which could be assigned as CLN9.[23]

Clinical Characteristics

The NCLs are fatal neurological diseases with manifestations restricted almost entirely to the central nervous system. The childhood forms are characterized by visual impairment leading to blindness (except CLN8), progressive psychomotor retardation, and severe epilepsy, finally leading to premature death.[1,2,7,24,25] The adult forms (adult CLN1, Kufs and Parry diseases) are characterized by psychological problems and/or dementia.[1,2,5,26] Depending on the type of NCL in question, the presenting sign, age of onset, and the length of the disease varies, e.g., the infantile CLN1 presents with psychomotor decline between 8-14 months of age, CLN2 presents with epilepsy between 2 and 4 years of age, and CLN3 presents with visual failure between 4 and 8 years of age. The typical clinical course, the temporal appearance of different symptoms, as well as the specific neurophysiological alterations in different forms of NCL are described in detail in a handbook of NCLs[27] and in recent reviews.[1,2,7,25] However, different mutations in a single gene can lead to a variety of phenotypes:[28] For example, mutations in the CLN1 gene can lead to the classical infantile onset disease, to a protracted juvenile form of the disease,[29,30] or to an adult onset variant of the disease.[26]

Neuronal Degeneration

One of the key features common to all forms of NCLs is progressive neurodegeneration in the neocortex and retina (retinal degeneration is not observed in CLN8 or adult onset NCLs), often also in the cerebellum.[2,27] The earlier the onset of the disease, the worse the neurodegeneration. At the time of autopsy, the infantile CLN1 brains typically weigh only 250-350 g[31] (Fig. 1). There are hardly any neurons left in the neocortex and large areas of the cortex are replaced by hypertrophic astrocytes.[31] The only surviving populations of neurons in the cortex are the giant Betz cells and occational pyramidal neurons in hippocampal CA1 subfield.[31] At the time of autopsy, the active period of neuronal degeneration has been passed and microglial activation has already declined. In contrast to the complete loss of cortical neurons, the subcortical and spinal neurons are well preserved despite marked intraneuronal storage. In the cerebellum, most Purkinje cells and the whole granule cell layer are destroyed.[31]

At the time of autopsy, the CLN2,[32] CLN5[33] and CLN6[34] brains are severely atrophic and typically weigh 500-700 g, 450-650 g, and 600-900 g, respectively. The neuronal loss in the cortex is not as extreme as in CLN1. Except CLN2, the overall cytoarchitecture of the cortex is preserved. Often laminar loss of neurons occur, particularly in layers III and V.[32-34] The remaining neurons in lamina III typically show meganeurites, that is, axonal enlargements filled with storage material.[32-34] In addition, there is severe astrocytosis and microglial infiltration in the cortex. The subcortical structures show moderate to pronounced storage but relatively modest neuronal loss. In the cerebellum, the number of Purkinje cells and granule cells are severely reduced, leading to an almost total depletion of the granule cell layer.

The CLN3 brains are moderately atrophic, and at the time of autopsy, they weigh 800-1000 g[35] (Fig. 1). There is variable depletion of neurons that may not be obvious. However, selective loss of stellate neurons in lamina II as well as loss of neurons in cortical lamina V, amygdala, and striatum has been observed.[36] The cortical neurons show ubiquitous storage of autofluorescent storage material and are morphologically abnormal. The neuronal loss is

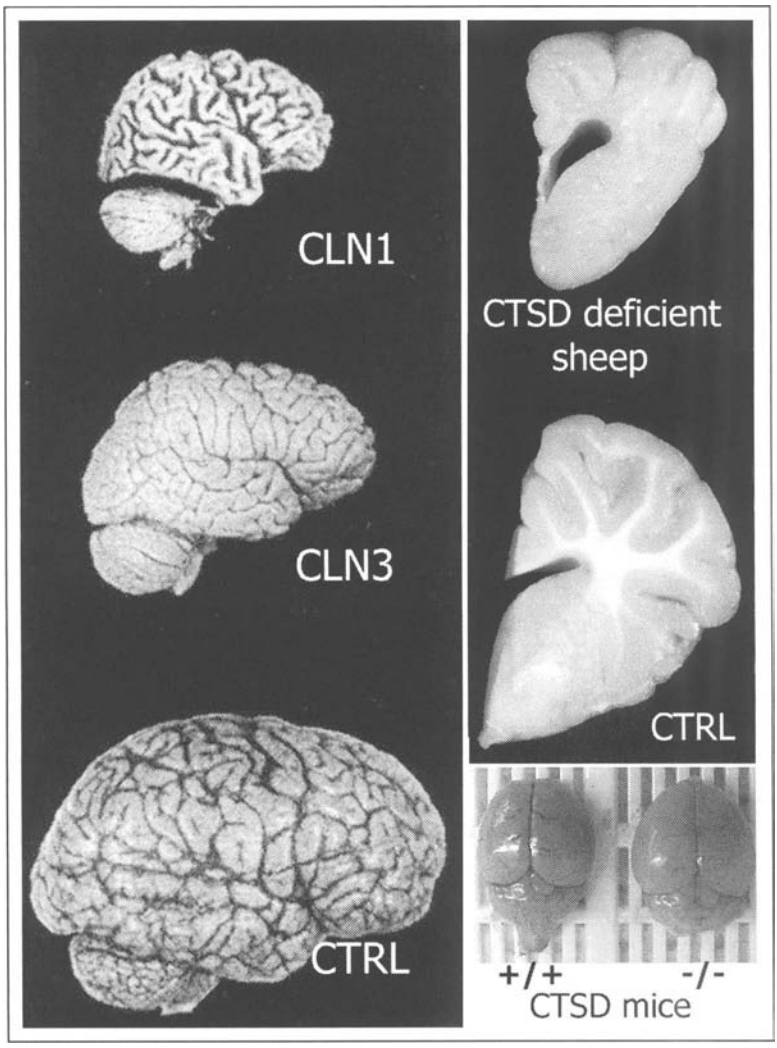

Figure 1. Brain atrophy in different forms of NCL. At the time of autopsy (usually 10-14 years of age) the CLN1 brains are extremely atrophic, weighing only 250-350 g. The CLN3 brains are moderately atrophic, weighing 600-900 g at death (15-40y). The normal brains (CTRL) weigh about 1200 g. The brains of a newborn lamb with cathepsin D deficiency (CTSD deficient sheep) weigh about 50% of normal (CTRL): the cortical areas are severely affected, while the brain stem is relatively preserved. In the cathepsin D knock-out mice (CTSD -/-), the brain weight is reduced compared to control mice (+/+), but the atrophy is difficult to observe visually.

associated with reactive astrocytosis and microglial activation. The cerebellar granule cell layer is severely affected, while the Purkinje cells are better preserved.

CLN8 brains are macroscopically normal at autopsy, and histochemical methods did not show neuronal loss in the cerebral cortex, except a patchy and almost complete depletion of pyramidal neurons in the hippocampal CA2 subfield.[37] The neurons show uniform storage throughout the cortex. The cerebellar granule and Purkinje cells are relatively well preseved. Despite this, MRI reveals cerebellar and brain stem atrophy already in early adulthood.[38]

As all patients with childhood NCLs suffer of severe epilepsy, it is of interest to note that, in contrast to the classical temporal lobe epilepsies, the neurons of the hippocampal CA1 subfield were relatively spared among all forms of NCLs. This data suggest that the death of hippocampal neurons is not a mere consequence of the frequent seizures, but rather reflects the underlying metabolic defect of the disease. A further interesting point is that in addition to principal excitatory neurons, the inhibitory neurons degenerate in all forms of NCL.[136]

Retinal atrophy has been observed in most forms of NCL, except CLN8 and the adult NCLs.[27] At the time of autopsy, the photoreceptor cells, bipolar cells, and the ganglion cells have been completely depleted and replaced by a gliotic scar in CLN1[39] and CLN3,[35] as well as in the advanced cases of CLN2[32] (and most likely also in CLN5 and CLN6). The retinal atrophy is a feature which is nicely replicated in several mouse models of NCL (see below). Apoptotic cell death has been observed in CLN3 knock-out mice.[40,41] In the cathepsin D knock-out mice two different cell death pathways were detected: apoptotic cell death with caspase activation was predominant in the outer nuclear layer, while nitric oxide mediated cell death was detected in the inner nuclear layers.[42] It remains to be seen whether the mechanisms of cell death are similar in the cortical and retinal neurons.

Neuronal Storage Material

The storage material in NCL diseases histochemically resembles ceroid and lipofuscin, which accumulates during normal ageing. The storage deposits are predominantly found in neurons, hence the name "neuronal ceroid-lipofuscinoses.". The NCL storage material can be distinguished from storage deposits found in other lysosomal storage diseases by their strong autofluorescence, histochemical properties, and distinct EM ultrastructure[2,43] (Fig. 2). The storage deposits are found in the cytoplasm of neurons (and other cells), and they are surrounded by a double membrane. These acid phosphatase positive storage granules are likely to represent the lysosomal compartment of the cell, but endosomal involvement in the storage process cannot be ruled out. In all forms of NCL, there is profound accumulation of the storage deposits in the neurons of the CNS, so that the neuronal cytoplasm is extended by the storage material[2,43](Figs. 2-4). In addition to neurons, macrophages show relatively high amounts of storage material. A smaller amount of storage deposits can be found in a number of tissues and cell types, including cardiomyocytes, kidney tubular epithelial cells, and hepatocytes.

The storage material is largely composed of protein,[44,45] and different forms of NCLs can be divided into two groups based on the identity of the main storage protein: those storing subunit c of the mitochondrial ATP synthase (subunit c) and those storing sphingolipid activator proteins (SAPs).[46] Subunit c is stored in most forms of NCLs, including CLN2, CLN3, CLN5, CLN6, CLN8, Kufs disease, and multiple animal NCLs.[33,34,37,46-48] SAPs A and D are stored in CLN1,[45] Parry disease,[23] cathepsin D deficient NCL sheep[10] and adult-onset Schnauzer NCL[49] (Fig. 3). The relationship of the stored proteins to the underlying genetic defects in the NCLs, as well as the role of the storage proteins in the pathogenesis of NCLs remain open.

The electron microscopic ultrastructure of the storage material has been, and still is, an important diagnostic tool for unclear cases of NCLs. The storage material is always osmiophilic but its electron microscopic fine structure varies: the storage deposits may have a granular, curvilinear, rectilinear or fingerprint type fine structure (Table 1). It seems that the type of stored compound determines the ultrastructure of the storage deposits: Subunit c storage is associated with either curvilinear, rectilinear or fingerprint type electron microscopic findings, while storage of SAPs is always associated with GRODs.[2,37,43]

Palmitoyl-Protein Thioesterase 1 (PPT1)

Mutations in PPT1 gene underlie CLN1.[8] PPT1 (EC 3.1.2.22) was originally isolated from bovine brain.[50,51] It is a soluble glycosylated enzyme of 37-39 kDa in molecular weight.[8,51,52] The 3D structure of PPT1 has been solved, showing that PPT1 is a globular monomeric enzyme with an α/β hydrolase fold and a catalytic triad of Ser^{115}, Asp^{223}, and His^{289}.[53]

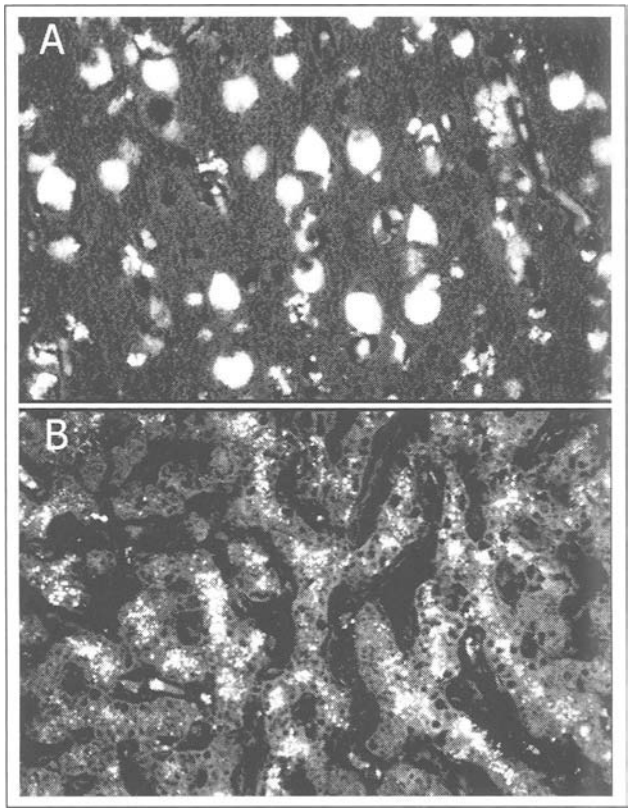

Figure 2. Storage of autofluorescent deposits. In adult onset Parry disease, (A) the autofluorescent storage material fills the neuronal perikarya throughout the cortex. B) Smaller amounts of autofluorescent storage deposits are also observed in liver hepatocytes. Courtesy of professor M. Elleder, Charles University, Prague, Czech Republic.

PPT1 removes acyl chains from S-palmitoylated proteins and CoA by hydrolyzing the thioester bond between the carboxyl group of the fatty acid and the sulfhydryl group of the cystein residue.[50] PPT1 has a broad neutral pH optimum, and a broad substrate specificity.[54] In vitro, PPT1 can depalmitoylate acyl chains linked to different proteins and peptides, including H-ras and neurospecific peptides originating from GAP-43 as well as rhodopsin.[50,54] Overexpression of PPT1 has been reported to decrease the palmitoylation-dependent binding of GAP-43 and H-ras to the plasma membrane, thus suggesting that these proteins could be in vivo substrates of PPT1.[55]

PPT1 is a ubiquitously expressed protein with highest enzyme activity in brain and testis.[51,56] In the brain, PPT1 protein is found in the neurons, and its expression increases during neuronal maturation and it temporally and spatially coincides with synaptogenesis.[57,58] In nonneuronal cells, PPT1 localizes to lysosomes and is mainly transported there via the classical Man-6-P pathway.[52,59] In neurons, PPT1 is also found in synaptic structures: in both synaptic vesicles and tightly associated with the synaptic membrane.[60,61] Further, PPT1 has been shown to be axonally targetted in mouse primary neuron cultures.[62] In a rat model of epilepsy, the level of PPT1 protein in synaptic junctions increased after seizures, possibly suggesting a role for PPT1 in synaptic plasticity and neuronal survival.[61] Compatible with this, PPT1 depleted cells have been reported to be prone to apoptosis.[55]

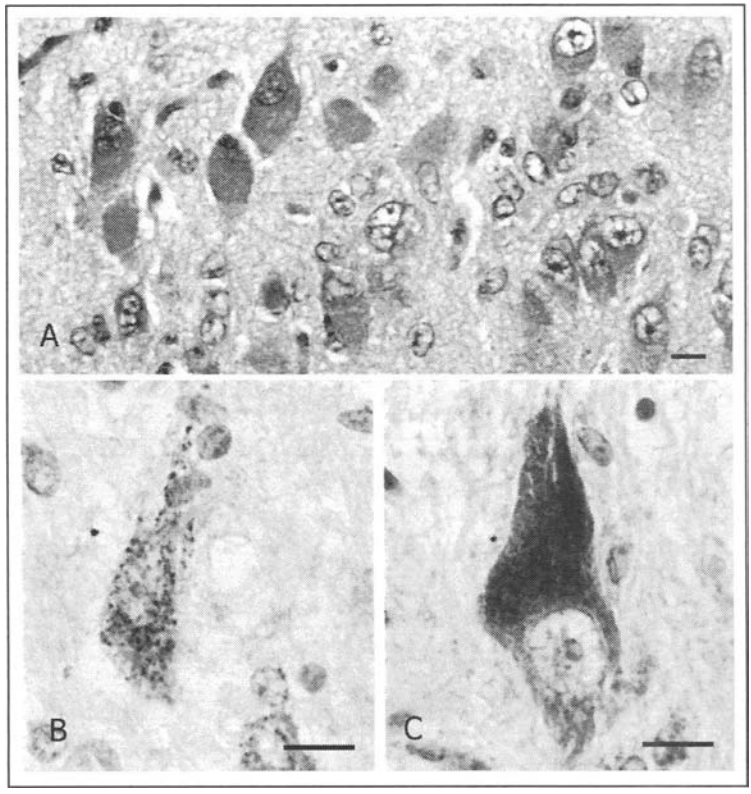

Figure 3. Histochemical properties of storage deposits. A) In cathepsin D deficient sheep, the neurons in the cerebral cortex harbor PAS-positive storage material. The neurons are also unevenly packed, reflecting the neuronal loss. B) Immunohistochemical staining of a normal sheep pyramidal neuron with antibodies against SAP D show a punctate lysosomal staining, (C) whereas in cathepsin D deficient sheep, the whole neuronal perikaryon stains with the antiserum. Scale bars, 10 μm.

In mammals, there are two further protein thioesterases in addition to PPT1: PPT2 and acylprotein thioesterase 1 (APT1). APT1 is a cytosolic protein with depalmitoylation activity against H-ras.[63] PPT2 is is a lysosomal thioesterase 27% identical to PPT1. The substrate specificity of PPT2 differs from that of PPT1, so that PPT2 is unable to hydrolyze palmitoylated proteins, and, thus, PPT1 and PPT2 are likely to perform nonredundant roles in thioester catabolism.[64]

Tripeptidyl Peptidase I (TPPI)

TPPI (EC 3.4.14.9) is an exopeptidase, the defects of which underlie CLN2.[9,65] TPPI is a lysosomal serine protease and the first mammalian member of the recently defined family of sedolisins, with Ser^{475} being the catalytically active nucleophile.[66-68] As many other lysosomal enzymes, it is synthesized as a preproenzyme and transported to lysosomes via the Man-6-P pathway. The inactive 66 kDa TPP proenzyme is proteolytically activated in the acidic environment of lysosome, resulting in a mature enzyme of 46-48 kDa in molecular weight.[67,69,70] Based on gel filtration experiments, it has been suggested that the functional unit of TPPI could be an oligomer consisting of six identical subunits.[71] There are five potential glycosylation sites in TPPI, all of which are utilized, the proper glycosylation of Asn^{286} being essential for folding and activity of the enzyme.[72,73]

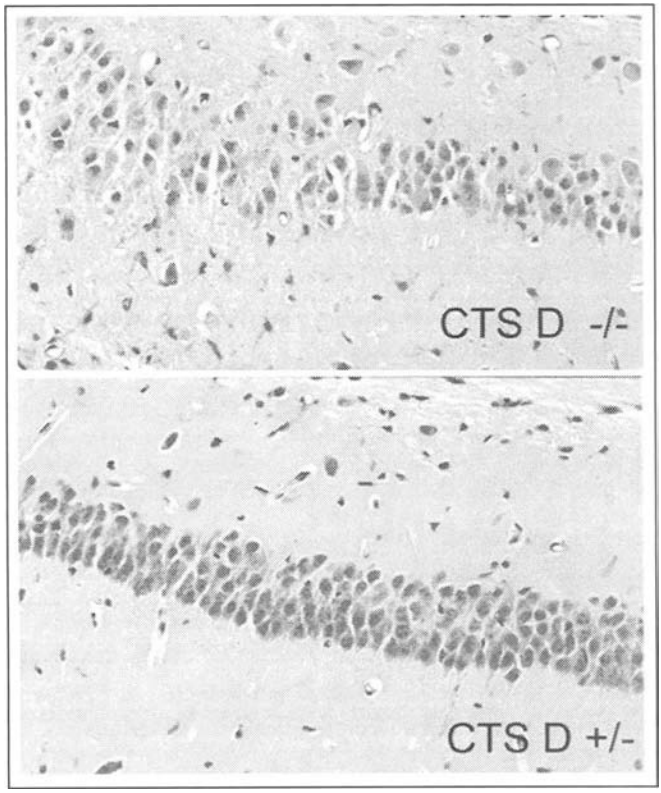

Figure 4. Hippocampal pathology observed with PAS staining. In the cathepsin D knock-out mice (CTSD -/-), the layer of pyramidal neurons is severely distorted in hippocampal subfield CA1 as compared to controls (CTSD +/-). In certain neurons, the cytoplasm is profoundly extended by the storage material.

In vitro, TPPI can cleave N-terminal tripeptides from a number of unfolded substrates with a free N-terminus and an uncharged amino acid in the P1 position, including peptide hormones such as angiotensin II and III, glucagon, substance P, and neuromedin B.[65,69,71,74,75] Recently, it was shown that cholecystokinin and neuromedin degradation in mouse brain is dependent on TPPI activity.[75] Thus, TPPI may have an important role in neuropeptide degradation in the CNS. In addition, TPPI may be involved in the degradation of subunit c, which accumulates in most forms of NCLs.[76]

As PPT1, TPPI is expressed in most tissues including brain.[71,77,78] In the brain, TPPI is expressed in both neurons and glial cells.[78] The amount of TPPI protein strongly increases in the developing rat brain after the first postnatal week,[79] and reaches the adult levels around two years of age in human brain.[78]

In addition to TPPI, there is another tripeptidyl peptidase in mammals, TPPII. While TPPI is a lysosomal enzyme, TPPII is a cytosolic one and it belongs to the subtilisin class of serine proteases, and therefore these enzymes are likely to have distinct physiological roles.[80]

Cathepsin D

A mutation in cathepsin D gene causes the congenital ovine NCL.[10] Cathepsin D (EC 3.4.23.5) is a lysosomal enzyme belonging to the pepsin family of aspartyl proteases. As TPPI, cathepsin D is synthesized as a preproenzyme, which is proteolytically processed during its

transport to the lysosome and activated in the acidic environment.[81,82] Proteolytic cleavage of the N-terminal propeptide results in formation of an active single chain form of the enzyme. In most species, this single chain form of cathepsin D is further processed and the mature enzyme is composed of two polypeptides, the heavy (30 kDa) and light (14 kDa) chains. Cathepsin D is also glycosylated. Its transport to the lysosome varies depending on the cell type, and both Man-6-P dependent and independent mechanisms may be used.[82-84] A fraction of cathepsin D is also secreted.[82,83]

Cathepsin D has a bilobed structure, with an active-site cleft between the two β-barrel domains. Both of these domains contain one of the catalytically important aspartic acid residues (Asp^{97} and Asp^{293} in mouse cathepsin D).[82,85] Cathepsin D has an acidic pH optimum, which varies from 3.5 to 5.0 depending on assay conditions and substrate used. Its activity can be inhibited by pepstatin A.[82] In addition to its proteolytic activity, cathepsin D has been implicated in many biologically important processes related to cell proliferation and apoptosis.[86-89]

Cathepsin D is expressed in all tissues including the brain, where it is abundant in neurons. In developing rat brain, the expression of cathepsin D mRNA was relatively constant. At protein level, the single chain form the cathepsin D was present already early during the embryonal development, and low levels of the 30 kDa form became visible after the first postnatal week.[79]

CLN3 Protein

The *CLN3* gene was identified in 1995, and it encodes a novel putative membrane protein predicted to have several transmembrane domains.[90] Technical obstacles and the difficult nature of CLN3 protein led to controversial results in the early localization studies. According to the present knowledge, CLN3 is a heavily glycosylated polytopic membrane protein of approximately 60 kDa in molecular weight, and it is a resident of the endosomal/lysosomal membrane.[90-93] The lysosomal targetting motifs of CLN3 protein were very recently identified, one being a conventional dileucine motif and the other being an unconventional [$M(X)_9G$] motif at the C-terminal tail of the protein.[94] However, it has been proposed that a fraction of CLN3 protein would be trafficked to lysosomes via the plasma membrane.[93] In neuronal cells, CLN3 protein has also been found in endosomes along the neuronal processes as well as in the synaptic region.[94,95] Interestingly, the targeting of CLN3 to neuronal processes was independent of the lysosomal targetting motifs.[94]

The function of CLN3 protein remains elusive, but clues to its function have been obtained from its yeast ortholog, Btn1p, which participates in acidification of the yeast vacuole, the equivalent of lysosome.[96,97] Deletion of the btn1 gene in *Saccharomyces cerevisiae* leads not only to elevated vacuolar pH but also to deficient arginine transport,[98] and therefore it has been suggested that CLN3 would have a role in controlling the lysosomal pH or, alternatively, transport across the lysosomal membrane.[99] In this context it is relevant, that the intralysosomal pH in CLN3 patient cells was elevated.[100] Other suggested functions for CLN3 include a role in the control of apoptosis.[101]

CLN5, CLN6 and CLN8 Proteins

The *CLN5* gene was predicted to encode a novel membrane protein with two transmembrane domains.[12] When overexpressed in BHK cells, the human CLN5 protein was localized to lysosomes.[102] Also, it was heavily glycosylated and secreted, suggesting that it is a soluble lysosomal protein of about 60 kDa in molecular weight. The predicted molecular weight of the protein is 46.3 kDa, but the deglycosylated human CLN5 protein was substantially smaller, about 35 kDa in molecular weight.[102] This indicated, that in the used overexpression system, the human protein was not translated from the first initiation methionine, but one of the alternative initiation sites was utilized.[102] When the human CLN5 protein was overexpressed in COS-1 cells, polypeptides of different sizes were synthesized, suggesting that in COS-1 cells, multiple initiation sites were used for the human CLN5 protein, and the longest form was

suggested to be membrane-bound.[104] Currently it is not known, whether the alternative initiation sites are used in vivo, e.g., depending on the tissue or developmental stage in question. The very recent characterization of the mouse CLN5 protein showed that it is a soluble lysosomal protein, the gene of which has only one initiation methionine.[104] The function of CLN5 protein remains to be elucidated, but it has been shown to interact with the CLN2 and CLN3 proteins in vitro.[103]

The most recently identified CLN gene is *CLN6*, encoding a previously unknown protein.[13,14] According to very recent data, CLN6 protein is a polytopic membrane protein of about 27 kDa in molecular weight and it resides in the ER.[105,106]

Mutations in the *CLN8* gene underlie the eighth form of NCL, the Northern epilepsy, also called progressive epilepsy with mental retardation (EPMR).[15] As with CLN5 and CLN6, CLN8 is a novel protein with an unknown function, and based on hydrophobicity characteristics, it is predicted to be a transmembrane protein. When overexpressed in COS-1 cells, the human CLN8 protein was found to be nonglycosylated and about 33 kDa in molecular weight. It was localized in ER and the ER-Golgi intermediate compartment.[107]

Animal Models

In addition to man, naturally occurring forms of NCL are found in domestic animals, including cat, dog, sheep, cow, horse, ferret, and mouse. Furthermore, a number of genetically engineered models of NCL diseases have been created in mouse, yeast, nematode, and, most recently, in fly. A summary of NCL animal models is represented in Table 2.

The South-Hampshire ovine NCL is the most extensively studied animal model of NCL. Pathologically, there is severe cortical and retinal atrophy and intraneuronal storage of autofluorescent material throughout the cortex in these sheep.[108-110] Based on developmental studies in this model, it was suggested that the neuronal loss may begin in the cerebral lamina IV. Also, it was in this model where subunit c storage was first discovered.[44] Presently it is known that the disease is caused by mutations in the sheep ortholog of CLN6 gene.[111] A further CLN6 sheep model has recently been reported in Australian Merino sheep, also showing intraneuronal storage of subunit c and progressive neurodegeneration in the cerebral cortex.[112] A congenital ovine NCL was discovered in Swedish white landrace sheep during the early 1990s.[113] This disease is characterized by very severe brain atrophy already at birth, and the storage deposits show a finely granular ultrastructure and SAP accumulation, thus resembling the human CLN1[10] (Fig. 3). However, a mutation in cathepsin D gene was identified as a cause of this disease.[10]

There is also a number of NCLs occurring in different breeds of dogs.[114] Subunit c and its vacuolar counterpart, the subunit c of vacuolar ATPase, compose the bulk of the storage material in most canine NCLs,[48] except the adult-onset NCL in Miniature Schnauzers, where the storage deposits exhibit a granular ultrastructure and SAPs accumulate.[49] The underlying genes have not been identified in any of the dog models of NCL.

There are two naturally occurring mouse models of NCL, the nclf[115] and mnd[15,116] mice, which have defects in genes orthologous to human CLN6 and CLN8, respectively, and show relatively mild disease phenotype. Furthermore, mice deficient in CLN1,[117,118] CLN2,[118] CLN3,[41,119,120] CLN5,[118] and cathepsin D[121] have been engineered via genetic manipulation. Generally, these mice replicate the key features of NCL diseases, including storage deposition and progressive degeneration of neurons in the cerebral cortex and retina.[41,117-120,122-124] However, the cortical neurodegeneration is essentially milder than in the corresponding human or large animal NCLs, and the lifespan of the mice is not severely reduced, except in CLN2 and cathepsin D deficient mice[118,124] (Fig. 1). Despite this, at least the CLN1, CLN3 and cathepsin D deficient mice have clear neurologic symptoms, including epileptic seizures. Therefore, these mouse models of NCL will provide a valuable tool to study the pathogenetic mechanisms and progression of NCLs. An excellent up-to-date review on the mouse models of NCL discusses the pathology and phenotypes in detail.[118]

Table 2. Animal models of NCL

	Gene	Onset	EM	Storage Proteins	Pathology and Presentation	References
Ovine NCL						
White Swedish landrace sheep	Cathepsin D	Congenital	GRODs	SAPs	Severe cortical atrophy, the brains weight about 50% normal at birth. Profound storage and clear neuronal loss. Enlarged ventricles. Tremor, muscle weakness, unable to survive.	10,113
South Hampshire sheep	*CLN6*	9-12 mo	FP, CL	Subunit c	Severe cortical and retinal atrophy, brains weigh about 60-70% at 13 mo. Profound storage and clear neuronal loss. Tremor, blindness.	44,47, 108-111
Merino sheep	*CLN6*	7-12 mo	FP, CL	Subunit c	Severe cortical and retinal atrophy. Storage throughout cortex. Visual impairment, behavioral changes, seizures.	112
Canine NCL						
Border collie, English setter	? (Recessive)	Early adult (1-2y)	FP, CL	Subunit c	Cortical atrophy, no retinal atrophy. Storage throughout cortex. Behavioral changes, convulsions.	48,114
Miniature Schnauzer	? (Dominant)	Adult (3-4y)	GRODs	SAPs	Retinal degeneration. Moderate storage throughout cortex. Behavioral changes, blindness.	49,114
Murine NCL						
CLN1	*CLN1*	Death by 7-10 mo	GRODs	?	Clear cortical atrophy. Storage of autofluorescent material. Motor problems, seizures	117,118, 134
CLN2	*CLN2*	Early death	?	?	Ataxia	118
CLN3, Three models	*CLN3, knock-out*[119]	Death by 20 mo[119]	FP	?	Slight cortical atrophy, progressive storage, motor problems.[119] Retinal degeneration but no obvious cortical changes at 17 mo, retinal storage, abnormal gait.[41]	40,41, 119,120
	CLN3, knock-out[120]		Membranous	?		
	CLN3, knock-in[41]	80% survive 12 mo[41]	FP	?		
Nclf-mouse	*CLN6*	Death by 12 mo	Membranous	Subunit c	Slight cortical atrophy, retinal degeneration and storage. Rear limb paresis	115
Mnd-mouse	*CLN8*	Death by 12 mo	Membranous	Subunit c	Slight cortical atrophy, retinal degeneration and storage. Motor problems, ataxia, blindness.	116,122, 123,133
Cathepsin D knock-out	*Cathepsin D*	Death at 25-27d	FP, GRODs	Subunit c	Slight cortical atrophy, retinal degeneration. Storage in hippocampus, retina. Motor problems, blindness, anorexia, seizures.	42,121, 124

Abbreviations: GRODs, granular osmiophilic deposits; CL, curvilinear profiles; FP, fingerprint profiles; RL, rectilinear profiles; subunit c, mitochondrial ATP synthase subunit c; SAPs, sphingolipid activator proteins

Furthermore, NCLs have been modeled in lower organisms, including yeast, nematode and fly. Studies on the function of the yeast ortholog of CLN3, btn1p, has given insight into the function of CLN3 as a potential regulator of lysosomal pH and transport[96-98,125](see above). CLN3 disesase has also been modeled in *C. elegans* but the phenotype of the deletion mutants does not seem to differ from that of the controls.[126] The visual system of *Drosophila* is used for studying disease genes and their modifiers. As there is retinal degeneration in most forms of NCLs, *Drosophila* might be a suitable model organism for NCL studies. PPT1 is evolutionarily conserved and the *Drosophila DmPpt1* is 55% identical to the human protein. Overexpression of the fly *Ppt1* lead to apoptotic cell death in the eyes of flies.[127]

Interestingly, a neurodegenerative disease with NCL-like features was recently reported in CLC-3 knock-out mice.[128-130] The acidification of synaptic vesicles is impaired in these mice, and they suffer of severe retinal and hippocampal atrophy. CLC-3 is a 110 kDa chloride channel of intracellular vesicles, mainly localized to synaptic vesicles.[128]

Pathogenetic Considerations

Despite the genetic heterogeneity, the NCLs show surprisingly uniform neuropathologic features, as discussed above. This implies, that there may be a final common metabolic pathway leading to NCL diseases. As the neurons are selectively affected in NCLs, this putative pathway must be essential in the normal development and survival of neurons. Recently, the first experimental evidence in this direction was obtained by showing a physical interaction of CLN5 protein with TPPI and CLN3.[103]

The role of the storage proteins in the pathogenesis of NCL diseases is not understood. It has been suggested that the storage deposits per se would be neurotoxic. This idea, however, is not compatible with the observation that the degree of storage does not correlate with neuronal death. SAPs, which accumulate in CLN1, are involved in degradation of glycosphingolipids, but this process appears to be normal in CLN1 fibroblasts. Based on the fact that subunit c is a mitochondrial protein it has also been proposed that mitochondrial dysfunction, possibly related to a defect in energy metabolism, would occur in NCLs.

One possible explanation of the neuron-specific disease phenotype is that the NCL proteins would have nonredundant roles in neurons, or, alternatively, neuron-specific substrates. As discussed above, TPPI appears to be needed for the degradation of certain peptides in brain, while in other tissues the lack of its activity is compensated by dipeptidyl peptidases.[75] Interestingly, there are a lot of palmitoylated proteins in neurons, that is, putative substrates for PPT1, and many of them localize to synapses.[131] Thus, a failure to depalmitoylate these potential substrates might lead to a failure in synaptic signalling, recycling, or assembly. Reversible palmitoylation of synaptic proteins is known to be an important regulator of synaptic function, albeit the depalmitoylating mechanism is unknown.[132] However, the palmitoylation of these neuronal proteins occurs on the cytoplasmic side of the plasma membrane, and therefore it is difficult to understand how PPT1 could possibly reach these substrates from the vesicular compartment where it resides. In addition to PPT1, CLN3 has been localized to synapses. As discussed above, in nonneuronal cells, CLN3 has been suggested to be involved in acidification of lysosomes,[97-100] while in neuronal cells, CLN3 might be involved in acidification of synaptic vesicles, similar to CLC-3. Also the possibility of altered neuronal trafficking has gained attention, and hints to this direction have recently been obtained from studies in a CLN3 yeast model.[125] In this context it is important that the transport pathway of PPT1 and CLN3 into the neurites is presently unknown.

Another line of thinking points out to the possible role of interneurons in the pathogenesis of NCLs. Certain classes of interneurons are depleted in several mouse and human NCLs.[133,134] This could contribute to development of an imbalance of the excitatory and inhibitory systems in the brain, which could have deleterious effects for neuronal survival. The GAD-65 autoantibodies recently found in the sera of CLN3 patients could potentially also affect the glutamate/GABA balance in the brains.[135]

In summary, it is crucially important to learn more about the neuronal behavior and functions of the NCL proteins in order to understand the pathogenetic mechanisms of these diseases. Understanding the mechanisms of neuronal death in NCL diseases will be likely to provide information on the generally important aspects of neuronal metabolism, survival, and ageing.

Acknowledgements

I would like to thank the personnel of the NCL-laboratory, Institute of Biomedicine, University of Helsinki, for their constructive comments on the manuscript, as well as professor Matti Haltia for the close collaboration and support on NCL-studies. The author was financially supported by the Academy of Finland (1207016) and the Helsinki University Research Funds.

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CHAPTER 9

Cholesterol Transport in Lysosomes

Judith Storch* and Sunita R. Cheruku

Abstract

Cellular cholesterol trafficking includes numerous individual processes, as cholesterol cannot only be obtained endogenously or exogenously, but also has many possible fates within the cell, including incorporation into membranes, metabolic conversion to a number of different products, covalent modification of specific proteins, regulation of gene expression, and efflux from the cell. Distinct trafficking pathways are thought to exist for de novo synthesized cholesterol and cholesterol obtained via the receptor-mediated endocytosis of low density lipoproteins. Exogenously obtained cholesterol must pass through the endosomal/lysosomal compartment prior to its trafficking to other subcellular sites, such as the endoplasmic reticulum, the Golgi apparatus, and the plasma membrane. The inherited disorder Niemann-Pick type C, in which abnormal cholesterol trafficking from the endo-lysosomal compartment leads to substantial cholesterol and glycolipid accumulatioin in lysosomes, is caused by defects in either of two genes that encode for proteins designated as NPC1 and NPC2. NPC1 is a multiple membrane spanning domain protein containing a sterol sensing domain similar to those found in several proteins involved in cholesterol homeostasis. NPC2 is a small intralysosomal protein that has been characterized biochemically as a cholesterol binding and transport protein. While there is abundant evidence suggesting a role for the NPC proteins in late endosomal/lysosomal trafficking of cholesterol, their precise functions and mechanisms of action remain to be discovered.

Intracellular Cholesterol Transport

Cells acquire cholesterol either by de novo synthesis in the endoplasmic reticulum or by internalization of cholesterol containing lipoproteins, particularly low density lipoprotein (LDL), via receptor-mediated endocytosis. Cellular cholesterol has several possible fates: incorporation into membranes, efflux to extracellular acceptors, or conversion to cholesteryl esters, bile acids or steroid hormones. In tissues that secrete serum lipoproteins, such as liver and intestine, cholesterol can be packaged into these particles and thereby leave the cell. In addition to these well appreciated fates, over the past few years the importance of cholesterol in cell signaling and regulation of intracellular vesicular trafficking has also become evident. For example, cholesterol modification of the Hedgehog protein is essential for its cleavage into the active peptide that signals through the Patched protein.[1] Moreover, the intracellular cholesterol pool regulates the production of the active forms of sterol response element-binding proteins (SREBPs), transcription factors that affect the synchronized expression of multiple genes involved in lipid metabolism.[2,3]

It is thus clear that intracellular trafficking events may play a key role in the eventual fates of endogenously synthesized and exogenous cholesterol, and in regulation of cholesterol efflux. The cellular responses that regulate the intracellular cholesterol pool, including downregulation of LDL receptor expression and de novo cholesterol synthesis, and upregulation of cholesteryl

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ester synthesis in the ER, are closely associated with LDL uptake.[4] Uptake of exogenous cholesterol by the cell involves endocytosis of cholesteryl ester-rich LDL via the well studied receptor mediated pathway, followed by fusion of the endosomes with the lysosomal compartment. In the endosome/lysosome compartment the cholesteryl ester core of LDL is hydrolyzed to unesterified cholesterol and free fatty acid. Cholesterol must leave this compartment to induce the homeostatic responses that regulate the cellular cholesterol pool. However, the pathways for trafficking of lysosomal cholesterol remain unknown.[5] Studies of Niemann-Pick type C disease, a fatal autosomal recessive disorder characterized by an accumulation of cholesterol in the lysosomes, have shown that trafficking of LDL derived cholesterol out of the lysosome is dependent on at least two proteins, NPC1 and NPC2.[6,7] Thus, there is considerable interest in understanding how cholesterol is transported within cells, and how lysosomal cholesterol transport is integrated with overall cellular cholesterol movement.

At steady state, most of the cellular free cholesterol (60-80%) is found at the plasma membrane,[8] where it plays an important role in the structural properties of the phospholipid bilayer. It is generally thought that cholesterol is trafficked from the plasma membrane to intracellular organelles where it is used for a variety of purposes including esterification, synthesis of bile acids, and synthesis of steroid hormones. The dynamic movement of plasma membrane cholesterol from the cell surface to intracellular organelles may occur only when needed or it can be a constitutive process that coexists with an efficient mechanism that returns cholesterol to the plasma membrane. While it is generally thought that cholesterol movement within the cell occurs by vesicular transport, cholesterol may also move through the cytosol by binding to soluble sterol-binding proteins.[2] However, the exact mechanisms that regulate the movement of cholesterol within the cell remain incompletely understood. Interestingly, it appears that distinct trafficking pathways exist for the intracellular transport of endogenously synthesized and exogenously derived cholesterol. A simplified scheme of intracellular cholesterol transport is shown in Figure 1.

Endogenously Synthesized Cholesterol Transport

Cholesterol synthesized in the ER is transferred to the plasma membrane in an energy dependent process[9] that is independent of the Golgi.[12] Even though the ER is the site of cholesterol synthesis, ER membranes maintain a low steady state level of cholesterol, suggesting the existence of efficient transport mechanism(s). Indeed, the transport of cholesterol from the ER to the plasma membrane is known to be a rapid process, with a $t_{1/2}$ of 10-20 min.[10]

A common method used to quantify cholesterol delivery to the plasma membrane is via extraction by extracellular cyclodextrin (CD). Cyclodextrins are water soluble cyclic oligosacchrides that have the capacity to rapidly remove cholesterol from cells by sequestering it in their hydrophobic cavity. During such efflux experiments, cells are equilibrated with ^{3}H-cholesterol before the addition of extracellular cyclodextrin. The extraction of labeled cholesterol is typically biphasic, with the rapid phase thought to be removal from the plasma membrane, and the slow phase corresponding to delivery from internal organelles. Sphingomyelinase treatment of cells has been shown to enhance the rate and extent of removal of cellular ^{3}H –sterol by CD, as the plasma membrane cholesterol is thought to be tightly associated with sphingomyelin.[11] Neufeld et al observed that the treatment of fibroblasts with CD following sphingomyelinase treatment resulted in a biphasic removal of cholesterol, with an initial rapid phase of depletion, 70% in 15 minutes, followed by a subsequent slower phase with a $t_{1/2}$ of 5-6 hours.[11] This biphasic kinetics of newly synthesized cholesterol efflux remains functional in the presence of agents that inhibit microtubule transport (colchicine, cytochalasin), lysosomal function (NH_4Cl), or protein synthesis (cycloheximide).[12] Despite the absence of a cycloheximide effect, however, caveolin has been shown to facilitate the movement of cholesterol from ER to the caveolae (caveolin rich domains) in the plasma membrane, from which it rapidly distributes within the plane of the bilayer.[13] It has been proposed that caveolin forms a complex with chaperone proteins, thereby delivering cholesterol from the ER to the plasma membrane without trafficking through the Golgi apparatus.[14]

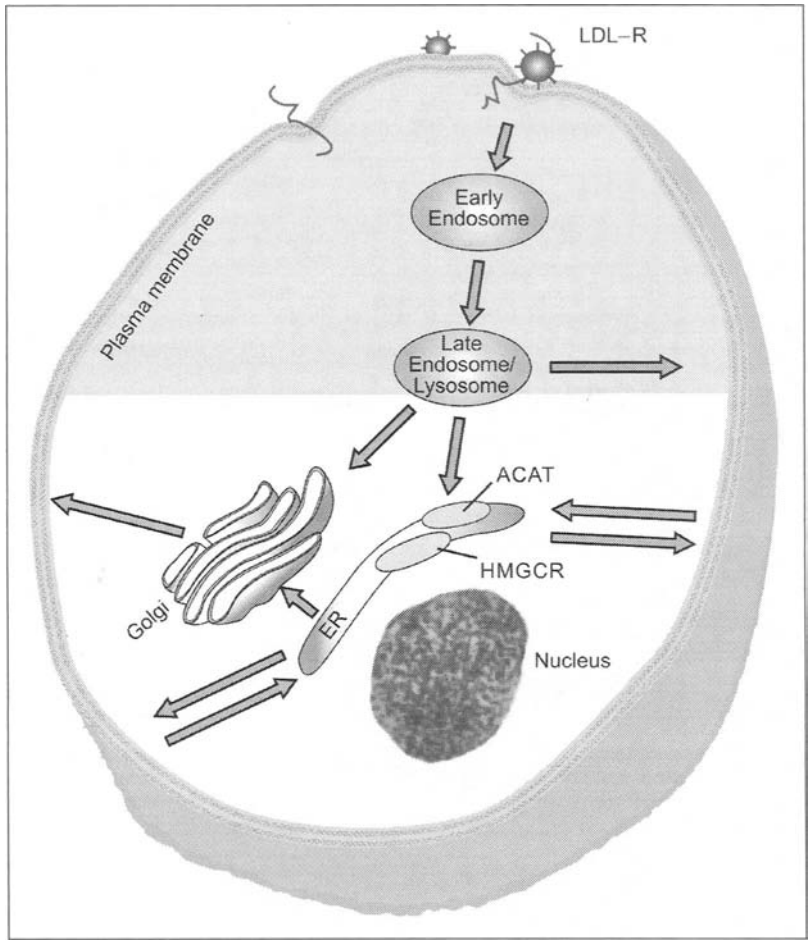

Figure 1. Cellular cholesterol transport. Routes of cholesterol trafficking between cellular compartments are shown by the yellow arrows. Separate pathways may exist for LDL-derived cholesterol and choleterol synthesized endogenously in the ER. Please see text for details. ER, endoplasmic reticulum. LDL-R, low density lipoprotein receptor. ACAT, acyl cholesterol acyltransferase. HMGCR, HMG-CoA reductase. A color version of this figure is available online at http://www.Eurekah.com.

A parallel route for delivery of newly synthesized cholesterol to the plasma membrane, involving a vesicle-mediated protein secretory pathway which passes through the Golgi, has recently gained attention. When Heino et al[15] treated cells with Brefeldin A, which causes a rapid disassembly of the Golgi network, it inhibited de novo synthesized cholesterol transport by 20%, suggesting a role for the Golgi in the delivery of newly synthesized cholesterol from the ER to the plasma membrane. Nevertheless, the majority of the de novo synthesized cholesterol uses transport routes independent of the Golgi.

Exogenously Derived Cholesterol Transport

In order for exogenously derived LDL cholesterol to activate cholesterol-mediated homeostatic responses, the cholesterol must be transported to and through the lysosomes.[25] Exogenous cholesterol is provided to the cell via the endocytic uptake of LDL, which is transferred

to the endosome/lysosome system where the cholesteryl ester core is hydrolyzed to release free cholesterol.[4] Under normal conditions the transfer of cholesterol from the lysosomes is an efficient process with very little accumulation of the endocytosed cholesterol in the lysosomal compartment, even in the face of an increase in the cellular cholesterol content of four fold.[17]

Endocytosed cholesterol leaving the lysosomes enriches both the plasma membrane as well as the ER, where it may be reesterified. In contrast to endogenously synthesized cholesterol, which primarily moves to the plasma membrane in a Golgi-independent process,[18] a number of experimental approaches provide strong evidence of a role for the Golgi in post lysosomal trafficking of LDL cholesterol to the plasma membrane. Coxey et al used filipin cytochemistry combined with freeze fracture electron microscopy to track the differential accumulation of LDL cholesterol in intracellular membranes.[19] Upon addition of LDL to normal human fibroblasts filipin-cholesterol complexes appeared in the cis/medial cisternae and in great amounts in the trans-Golgi cisternae. Based on these relative cholesterol enrichments, the authors speculated that the cholesterol in the trans-Golgi vacuoles, localized between the trans face of the Golgi stacks and the plasma membrane, could be a potential means for transport of the lysosomal cholesterol to the plasma membrane, which contains high levels of cholesterol, and that cis/medial Golgi cisternae could be involved in the transport of cholesterol from lysosomes to the relatively cholesterol-poor endoplasmic reticulum. Whether such differential trafficking of Golgi cholesterol pools occurs, remains unknown. Studies in which Brefeldin A was used to disrupt the Golgi provide additional evidence for the role of the Golgi complex in distribution of lysosomal cholesterol. In treated cells, transport to the plasma membrane was blocked, whereas lysosomal cholesterol transport to the ER was still observed, as the Golgi was capable of fusing with the ER.[11]

As mentioned above, the cholesterol released from the lysosomes reaches the ER, however, it is not yet established whether the lysosomal cholesterol moves directly to the ER or must first pass through the plasma membrane. Several studies have addressed this question, with somewhat varying results. Lange et al loaded the endocytic compartment of rat hepatoma cells at 18°C with [^{3}H] cholesteryl linoleate-labeled LDL and then chased at 37°C. At 18°C very little hydrolysis of [^{3}H] cholesteryl linoleate occurs, whereas the subsequent 37°C chase causes rapid hydrolysis. Free [^{3}H] cholesterol at the ER was measured by its appearance in cholesteryl oleate and palmitate esters. They found that almost all of the released [^{3}H] cholesterol from the lysosomes moved immediately to the plasma membrane without any lag, and the kinetics of plasma membrane appearance paralled the hydrolysis of [^{3}H] cholesteryl linoleate. In contrast, the reesterification of the released [^{3}H] cholesterol showed a characteristic lag of 0.5-1 hour, even as the rate of hydrolysis of the [^{3}H] cholesteryl esters was leveling off, suggesting that the ER-bound cholesterol passes through an intermediate compartment, such as the plasma membrane, prior to esterification in the ER.[20] Neufeld et al found that 2-hydroxypropyl-β-cyclodextrin, the above mentioned extracellular acceptor of cholesterol, blocked esterification of LDL-derived lysosomal cholesterol by approximately 70%, suggesting that the majority of endocytosed cholesterol is mobilized from the lysosomes to the ER via a plasma membrane mediated pathway, while the residual 30% of LDL cholesterol was transported via a plasma membrane independent pathway.[11]

Studies by Underwood et al also suggest that the bulk of the LDL cholesterol is mobilized directly to the plasma membrane, however they further propose that an additional, vesicular pathway delivers LDL cholesterol from lysosomes to the ER, independent of residence in the plasma membrane. They first used hydrophobic amines to cause lysosomal accumulation of cholesterol, and showed that LDL cholesterol transport to the esterification enzyme acyl cholesterol acyltransferase (ACAT) was blocked, without blocking the movement of cholesterol from lysosomes to plasma membrane, or the movement of plasma membrane cholesterol to ACAT. Second, LDL cholesterol transport to ACAT was found to be normal in a Chinese hamster ovary cell mutant that is defective in plasma membrane to ACAT movement of cholesterol; the near normal levels of LDL- [^{3}H] cholesterol esterification in these mutant cells suggests that LDL cholesterol must be transported from lysosomes to ER via a route that bypasses

the plasma membrane. Third, they measured the kinetics of LDL- [^{3}H] cholesterol versus cellular [^{14}C] cholesterol incorporation into cholesteryl esters, and found that LDL cholesterol is not diluted by the plasma membrane pool before reaching ACAT.[21] Other studies by Lupu et al[22] indicate that trafficking of LDL-cholesterol from lysosomes to ER may require an intact intermediate filament network since the human adrenal tumor cell line SW13, which lacks detectable vimentin, transports LDL cholesterol normally from lysosomes to plasma membrane but does not mobilize LDL cholesterol to ACAT in the ER.[22] Thus, studies to date suggest the existence of more than one post-lysosomal trafficking pathway for exogenously derived cholesterol, and indicate further that the general idea of the plasma membrane cholesterol pool as the source of all intracellular membrane cholesterol may not be strictly the case.

Mechanism of Cholesterol Transport from Lysosomes

Cholesterol esters derived from LDL are hydrolyzed inside the endosome/lysosome compartment by specific acid hydrolases. It is likely that the liberated free cholesterol, which like its parent ester has limited aqueous solubility, intercalates into lysosomal membranes, and/or is bound by intralysosomal proteins. It is currently not known what the rate-limiting step for cholesterol movement out of the lysosome might be, although possibilities may include the aqueous diffusion of cholesterol from the limiting lysosomal membrane to other organelles, vesicular trafficking of lysosomal membrane cholesterol to other subcellular locales and/or the involvement of specific lysosomal and perhaps cytosolic proteins.

Brasaemle et al found that free cholesterol released from LDL cholesteryl esters was rapidly transported to the plasma membrane, within minutes.[23] This rate of transport implies that the aqueous diffusion of cholesterol from lysosomes to the plasma membrane is unlikely, as aqueous diffusion of cholesterol between membranes is quite slow,[24] presumably because of the slow off-rate of cholesterol from phospholipid bilayers. Nevertheless, if the rate-limiting step in the overall movement lies in delivery of cholesterol to the limiting lysosomal membrane, or the transbilayer movement of cholesterol within this membrane, then aqueous diffusion in the cytosol could still be involved but not be the limiting event under normal circumstances. It is well known that the lysosomes are the site of lipid and protein catabolism, in contrast to the ER where the proteins and lipids required for vesicle formation are provided by de novo synthesis. Therefore it is improbable that lysosomal cholesterol egress occurs by vesicle budding, as the lysosomal membrane lipids would be depleted thereby affecting membrane integrity, and this has not been reported.[25] Thus, the most likely mechanism for the transport of lysosomal cholesterol would seem to involve one or more cholesterol binding and/or transfer proteins.

The accumulation of LDL-derived cholesterol in lysosomes of NPC cells suggests that movement of cholesterol out of the lysosomes depends upon the normal functioning of both proteins found to be mutated in NPC disease, NPC1 and NPC2. Additional endolysosomal or cytosolic proteins may also be involved in the lysosomal cholesterol transfer process, however it is likely that the NPC1 and NPC2 mediated pathway(s) is the rate-limiting route for normal trafficking of lysosomal cholesterol.[26]

Lysosomal Cholesterol Transport Candidates: Niemann-Pick C Disease

Niemann-Pick C disease is a rare autosomal-recessive disorder that affects diverse ethnic groups. NPC is a complex lipid storage disease characterized by the accumulation of unesterified cholesterol and glycolipids in the endosomal/lysosomal system.[26,27] Pentchev and coworkers[28] observed that fibroblasts from NPC patients were impaired in the esterification of LDL derived cholesterol, which typically occurs in the ER. Large amounts of cholesterol were found to accumulate in the lysosomal compartment, as expected. Nevertheless, in cells from patients with NPC disease, both uptake of LDL cholesterol and hydrolysis of cholesteryl esters occur normally. Following LDL uptake in affected cells, Coxey et al observed an increase in cholesterol enrichment in *trans* Golgi cisternae and a decrease in cholesterol accumulation in *trans*-Golgi vacuoles, relative to normal cells.[19] These results suggest that in NPC disease the lysosomal

cholesterol can be transported to the *trans* Golgi, whereas subsequent movement to *trans* Golgi vacuoles is either defective or perhaps delayed. It is likely that such defective trafficking of unesterified lysosomal cholesterol underlies the fact that expected downstream effects of LDL-cholesterol on cellular homeostatic responses are inhibited in the NPC cells.[29] In particular, the normal suppression of de novo cholesterol synthesis and LDL uptake are diminished.[29]

Genetic studies have identified two complementation groups in NPC patients.[30] The major disease locus, NPC1, maps to chromosome 18q11 and is responsible for 95% of NPC cases.[31] The minor disease locus, NPC2, maps to chromosome 14q24.3 and is responsible for 5% of cases.[7] In studies to date, the two complementation groups show indistinguishable cellular and biochemical characteristics, suggesting that the two gene products, NPC1 and NPC2, may function in a coordinated manner. There is abundant evidence that suggests a role for the NPC proteins in late endosomal/lysosomal transport of cholesterol, however their precise functions at the cell and molecular levels have not yet been determined.

NPC1

The gene for NPC1 encodes an approximately a 4.9kb mRNA that produces a 1278 amino acid protein. Using genomic organization analysis it has been shown that the NPC1 gene contains 25 exons ranging in size from 74 to 788 nucleotides and introns ranging from 0.097 to 7kb in size.[32] Numerous mutations, including nonsense and missense mutations, insertions, deletions and duplications, have been described in patients with NPC1 disease.[31,33-35] Mutations are found throughout the gene, suggesting that there is not a single functionally critical protein domain. However, Greer et al observed that a small cluster of mutations seen in the cysteine rich domain in the carboxy-terminal third of the protein are found in residues that are conserved between the various NPC1 orthologues.[34]

NPC1 has been shown to be a membrane glycoprotein that localizes to lysosome-associated membrane protein (LAMP) positive organelles, presumably endosomes and lysosomes.[36,37] Neufeld et al, through immunocytochemical studies, have demonstrated that the NPC1 gene product resides in a novel set of lysosome-associated membrane protein-2 (LAMP2)(+)/ mannose 6-phosphate receptor (-) vesicles that could be distinguished from cholesterol enriched LAMP2(+) lysosomes.[36] Further, Higgins et al have also demonstrated that the NPC1 colocalizes primarily with Rab9GTPase, a component of vesicles involved in late endosome –to-trans-Golgi network (TGN) transport, and that in the steady state NPC1 resides primarily in the late endosomes.[37] Both these studies showed that drugs such as U18666A and progesterone, that block cholesterol transport out of lysosomes, also lead to a redistribution of NPC1 to cholesterol rich lysosomes.[36,37] This suggests that in normal cells a transient interaction between NPC1 (+) organelles and cholesterol rich lysosomes is required to relocate cholesterol from the lysosomes to other cellular sites involved in so-called retrograde transport.[36] It is noteworthy that in addition to the cholesterol trafficking defect, *npc1*$^{-/-}$ cells cannot efflux endocytosed sucrose,[36] and have an impaired sorting of the lysosomal multifunctional receptor (IGF2/MPR).[38] This indicates that the retrograde transport of proteins and cargo from the late endosomes to the trans-Golgi network is generally disturbed in NPC1 deficient cells.

The level of NPC1 protein in cells appears to be feedback-regulated. Watari et al observed that the treatment of cells with progesterone, which induces a phenocopy of the NPC disease, caused a three-fold increase in NPC1 mRNA and protein levels with no effect on NPC1 promoter activity.[42] Thus it appears that conditions that block NPC1 action trigger a compensatory process which leads to increased stability of NPC1 mRNA and, hence, an increased NPC1 protein level.

Topological analysis of NPC1 protein reveals several motifs, each with a potentially important role in the retrograde transport of lysosomal cargo. The highly conserved N-terminus of the protein contains a leucine zipper motif and is called the NPC1 domain.[31] Mutations of the conserved cysteine residues in the leucine zipper motif disrupt the cholesterol mobilizing activity of the protein.[39,40] Beyond the NPC1 domain lie the 13 membrane spanning domains, with

sequences showing a strong homology to the sterol sensing domains (SSD) of the sterol response element binding protein cleavage activating protein (SCAP), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG coA reductase) and the Hedgehog signaling protein Patched.[31,41] Mutations in the sterol-sensing domain also inactivate the protein.[39] Downstream of the SSD lies the aforementioned cytoplasmic loop with 8 cysteine residues.[41] Several point mutations in this loop have been identified in NPC patients; these mutations inactivate the protein but do not affect the trafficking of NPC1 to the lysosomes.[34] The NPC1 C-terminus contains a dileucine motif which is critical for proper targeting to late endosomes, as a deletion of this motif caused the protein to be retained in the ER.[40]

The mechanism by which the NPC1 protein functions in retrograde transport of cholesterol and other lysosomal cargo is not yet known. A role for NPC1 as a general permease has been contemplated due to its structural homology to the prokaryotic resistance-nodulation-division (RND) protein. The RND protein participates in the efflux of lipophilic compounds such as bile salts and fatty acids, from the cytosol of Gram-negative bacteria.[43] The expression of human NPC1 in *E.coli* was used to examine its potential function as a molecular pump. Transport activity for fatty acids but not cholesterol or cholesteryl esters was reported, although potential confounding by bacterial membrane properties must be considered, and a distinction between binding and transport was not clearly made.[43] Thus, the relationship of NPC1 to cholesterol unloading is not yet understood at the organelle and protein levels.

Interestingly, a homologue of the NPC1 protein, NPC1-L1, was recently shown to be involved in the intestinal absorption of dietary cholesterol,[44,45] suggesting further that this family of SSD-containing proteins plays a specific role in cellular cholesterol trafficking. Like the NPC1 protein, the exact mechanism of action of NPC1-L1 remains unknown.

NPC2

About 5% of NPC cases have been attributed to mutations in the NPC2 gene. In 2000 Naureckiene et al[7] demonstrated that the previously identified HE1 protein was the product of the second gene of Niemann-Pick C disease. The HE1/NPC2 gene is located on chromosome14q24.3 and contains five exons.[7] HE1 was shown to be an ~18 kDa soluble glycoprotein, and subcellular fractionation studies demonstrated that it resides in the lysosomal lumen. Further, HE1/NPC2 was shown to undergo the classic mannose-6 phosphate modification whereby soluble proteins that acquire this posttranslational modification are recognized by the mannose 6 phosphate receptor, and such newly synthesized proteins are thereby diverted from the secretory pathway to the endolysosomal system. The NPC2 mRNA is translated into a 131 residue protein that is ubiquitously expressed, with high abundance in the testis, kidneys and liver.[7]

HE1 was characterized as a cholesterol binding protein present in mammalian epididymal fluid.[46] Purified HE1 bound cholesterol with high affinity (K_d = 2.3 uM) and a 1:1 stoichiometery. Recently Friedland et al[47] examined the ligand binding properties of NPC2 using dehydroergosterol (DHE), a naturally occurring fluorescent sterol that is structurally similar to cholesterol. Since NPC2 is a lysosomal protein they also measured the pH dependence of ligand binding. The DHE dissociation constants obtained were 0.19 ± 0.07 μM and 0.66 ± 0.39 μM at pH 5.0 and 7.0 respectively, consistent with the hypothesis that NPC2 binds cholesterol in the endolysosomal compartment.

The crystal structure of the unliganded form of bovine NPC2 (bNPC2) has been recently characterized by Friedland et al, revealing unique properties of the cholesterol binding site.[47] The structure of bNPC2 (Fig. 2) has an Ig-like β- sandwich fold consisting of seven β-strands which are arranged in two β-sheets. The first sheet contains three β strands and the second sheet has four β strands; a short half turn α-helix connects the fourth and the fifth β strands. Surprisingly, the structure of apo bNPC2 lacks any large cavity, tunnel or surface pocket that would accommodate a cholesterol molecule. Other sterol binding proteins such as elicitin and sterol esterase have a large hydrophobic cavity that is present in the absence of a ligand.

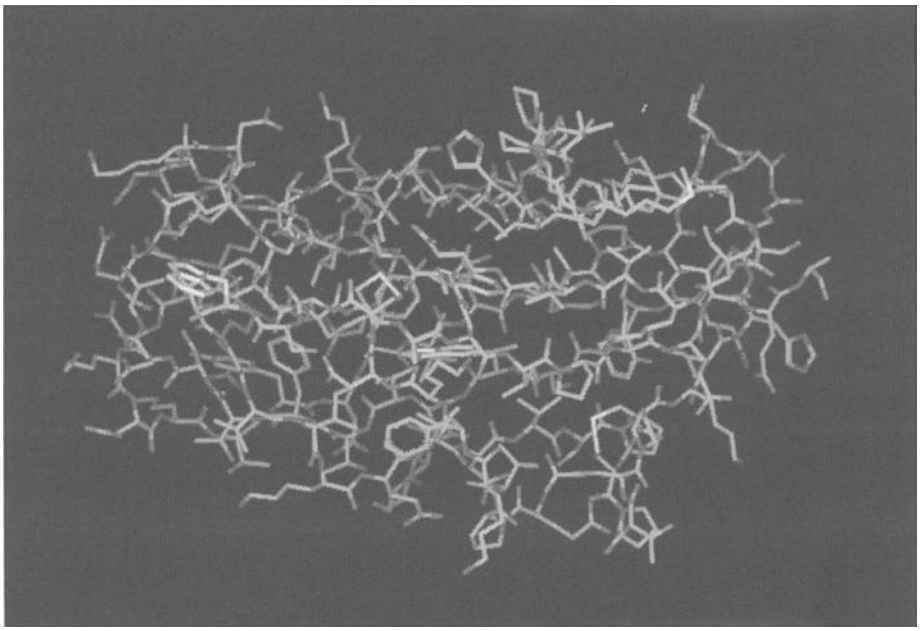

Figure 2. Structure of bovine apo-NPC2.[47] Backbone structure of bNPC2, with its 3 Trp side chains highlighted in yellow. Human NPC2 contains only 2 Trp residues, at positions 109 and 122. These two are identical with the corresponding residues in bNPC2, and both are seen to be in close approximation and facing the interior of the NPC2 protein. The fact that cholesterol quenches hNPC2 tryptophan suggests that it binds, at least in part, at an interior site. A color version of this figure is available online at http://www.Eurekah.com.

Although a tertiary structure of holo NPC2 is not yet available, several features implicate the hydrophobic interior of the protein as the binding site for cholesterol. The hydrophobic core of the protein is not densely packed but contains three small cavities, with volumes ranging from 36 to 84 $Å^3$. Two of the small cavities are connected by a gate formed by two aromatic residues, Phe-66 and Tyr-100. Although the combined volume of the three cavities, 158 $Å^3$, is too small to accommodate a 741 $Å^3$ cholesterol molecule, the overall shape of the cavities matches well with a space filling model of a cholesterol molecule.[47] Studies from our laboratory using the intrinsic tryptophan fluorescence of the human NPC2 protein also provide evidence that NPC2 binds cholesterol in an interior pocket. At present therefore, it is hypothesized that for cholesterol to bind NPC2, the two β-sheets move apart and the gate opens, forming a single interior tunnel that is sufficiently large to accommodate the NPC2 ligand.

The presence of a cholesterol binding site in the hydrophobic core of the NPC2 protein is further strengthened by mutagenesis studies conducted by Ko et al[48] Three point mutations, F66A, V96F and Y100A, which are located in close proximity to the each other in the hydrophobic region of NPC2, showed a reduced ability to bind cholesterol and were unable to restore normal levels of cholesterol in *npc2*$^{-/-}$ fibroblasts. This strongly suggests that cholesterol binding is essential for the function of NPC2 protein, and that binding occurs in an interior hydrophobic site.

What are the functional implications of cholesterol binding by NPC2? It could be that cholesterol binding is necessary for the transfer of endo/lysosomal cholesterol. By using the endogenous tryptophan fluorescence of NPC2, we have examined the transfer of cholesterol from purified human NPC2 to model membranes. The basic principle of the method is that

upon cholesterol binding to NPC2, the tryptophan signal is quenched; upon subsequent addition of acceptor phospholipid vesicles, the rate of transfer of cholesterol from the NPC2 protein to the membranes may be monitored directly by the increase in tryptophan fluorescence over time. To determine the mechanism of cholesterol transfer from NPC2 to membranes, the rate of cholesterol transfer was analyzed as a function of acceptor vesicle concentration and acceptor vesicle phospholipid composition. In this way[49] we were able to distinguish between an aqueous diffusion-mediated transfer mechanism, and one in which cholesterol transfer occurs during NPC2-membrane interactions.

To directly examine the possibility of collisional transfer of cholesterol, the theoretical number of collisions between egg phosphatidylcholine membrane vesicles and NPC2 was varied by increasing the concentration of these small unilamellar acceptor vesicles (SUV). The rate of cholesterol transfer from NPC2 increased in proportion to the frequency of NPC2-membrane interactions, suggesting a collisional mechanism of transfer. When anionic phospholipids (25 mol% phosphatidylserine (PS), phosphatidyl inositol (PI), bis(monoacylglycero)phosphate) (BMP; also termed lyso bis phosphatidic acid), or cardiolipin (CL)) were incorporated into the acceptor vesicles the cholesterol transfer rate was markedly increased relative to zwitterionic SUVs. The absolute cholesterol transfer rates obtained were several orders of magnitude faster than reported off-rates of cholesterol from NPC2 into aqueous media.[48] These results suggest that NPC2 binds cholesterol and can then transport it to the lysosomal membrane. It is envisioned that the delivery of NPC-bound cholesterol can occur either to the phospholipid bilayer, after which the cholesterol could reach a putative transmembrane transporter via lateral diffusion in the plane of the membrane. Alternatively, holo-NPC2 could interact directly with a lysosomal membrane protein (such as NPC1), which then directs the postlysosomal export of cholesterol. Either of these mechanisms is consistent with the finding of Ko et al[48] who showed that NPC2 rapidly clears the accumulated cholesterol in the *npc2* $^{-/-}$ fibroblasts.

The transport of cholesterol out of the lysosome may also involve the participation of a third protein, MLN64. MLN64 contains a StAR-related lipid transfer domain (START), and is localized to the late endosome/lysosomal compartment.[50] Based on these associations, it has been speculated that MLN64 may act in concert with NPC1 and NPC2 to effect cholesterol transport out of the lysosome. However, it was recently reported that in mice with targeted mutations of the MLN64 START domain only minor alterations in cholesterol metabolism and cellular distribution were found.[51] Another potential participant in intra-lysosomal trafficking of cholesterol is the unique phospholipid, bis (monoacylglycerol) phosphate (BMP). BMP is present in high concentrations in the internal membrane network within endo/lysosomes, and the treatment of BHK cells with an anti-BMP antibody resulted in cholesterol accumulation in late endosomes.[38] The abovementioned rapid transfer of cholesterol from NPC2 to BMP-containing model membranes suggests that this unusual lipid may serve as a localizing site for the interaction of NPC2 with acid hydrolase-liberated cholesterol.

It is likely that lysosomal cholesterol binds to the NPC2 protein and is transferred to the limiting lysosomal membrane, where it is subsequently exported out of the lysosome and is able to regulate activities that maintain normal cellular cholesterol levels. Frovlov et al[52] suggest that NPC1 and NPC2 function in the transfer of free cholesterol to both the mitochondrial sterol 27-hydroxylase and the ER/ Golgi-localized cholesterol 25-hydroxylase enzymes. They propose that in NPC disease, the failure to generate appropriate levels of 25 and 27- hydroxy cholesterol prevents feedback inhibition of SREBP dependent gene expression, e.g., HMG coA reductase and LDL receptor, and prevents the activation of LXR-regulated pathways, e.g., expression of ABCA1, a plasma membrane cholesterol efflux protein. Thus, the failure to inhibit cholesterol synthesis and the continued uptake of cholesterol via the LDL receptor, accompanied by impaired ABCA1-mediated cholesterol efflux, results in a massive accumulation of intracellular cholesterol.

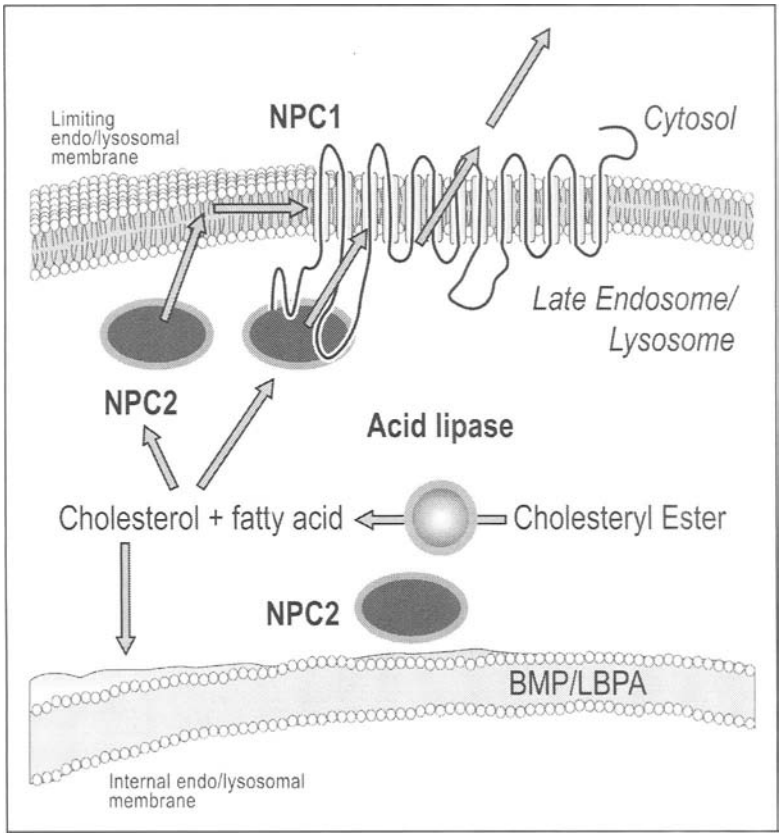

Figure 3. Hypothetical model of cholesterol efflux from the lysosome. Please see text for details.

Concluding Remarks

The mystery of lysosomal cholesterol transport has been partially unraveled with the identification of the NPC1 and NPC2 proteins. However the precise functions of these proteins at the cell and molecular levels, as well as their relationship to each other and, potentially, to other proteins, remains unclear. A hypothetical model of cholesterol efflux from the lysosome, meant to serve simply as a potential framework for further endeavors, is depicted in Figure 3. In this model, cholesteryl ester derived from LDL is hydrolyzed by acid lipase. BMP, enriched in intralysosomal membranes, may serve as a localizing site for the interaction of NPC2 with hydrolase-liberated free cholesterol. The unesterified cholesterol is bound by NPC2 protein within the lumen of the endo/lysosomal compartment. NPC2-bound cholesterol is then delivered to the limiting organellar membrane via direct interaction of the protein with the membrane, perhaps followed by lateral diffusion to membrane-bound proteins, or by direct interaction with membrane proteins, potentially NPC1. NPC1 then participates in the directed efflux of cholesterol from the endo/lysosomal compartment.

In the coming years, the elucidation of normal mechanisms of lysosomal cholesterol trafficking, as well as the functions of NPC1 and NPC2 proteins and their roles in cholesterol homeostasis, will enable us to understand the pathophysiology of NPC disease, thereby promoting the development of effective preventive and therapeutic strategies.

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CHAPTER 10

Therapy of Lysosomal Storage Diseases

Ulrich Matzner*

Abstract

Lysosomes are membrane-surrounded organelles which are present in all nucleated mammalian cells. They function to degrade both intra- and extracellular macromolecules to low molecular components that are transported to the cytoplasm for reutilization in the biosynthetic pathways of the cell. Up to now more than 50 different lysosomal hydrolases catalyzing the degradation of proteins, lipids, nucleic acids and carbohydrates have been identified. The normal function of the lysosomal compartment also depends on a variety of proteins without hydrolytic activity such as cofactors, transporters, ion channels and nonlysosomal proteins engaged in the posttranslational activation and targeting of lysosomal enzymes. Functional deficiencies, both of hydrolytic or nonhydrolytic polypeptides can result in lysosomal storage diseases (LSDs) which are characterized by the intralysosomal deposition of macromolecules and a multisystemic phenotype leading to early death. Most LSDs appear in severe, early onset forms and milder, late onset variants. This hetereogeneity seems to be due to subtle differences in the residual amount of active protein implying that even a modest increase of the protein level might have beneficial effects on the disease. Consequently, most therapies for LSDs attempt to supply deficient cells with the active counterpart of the defective protein. Such enzyme augmentation therapies contrast with substrate reduction therapies which try to reduce the accumulated storage material by nonenzymatic means. The evaluation of the potentials, limitations and risks of therapeutic strategies in cell culture and animal models for LSDs has been fundamental for the implementation of clinical trials which finally led to the clinical realization of certain treatment concepts.

Lysosomal Storage Diseases

Lysosomal storage diseases (LSDs) are a group of more than 40 inherited metabolic disorders (Table 1 and Chapter 6) which are macroscopically characterized by the deposition of storage material in the lysosomal compartment of cells.[1] The granules consist of undegraded or partially degraded macromolecules which accumulate as a consequence of a reduced or missing activity of a polypeptide involved in the normal function of the lysosome. The metabolic defect causes the dysfunction and possibly the loss of distinct cell populations and the manifestation of a complex disease phenotype which usually affects many tissues. Although the disease manifestations vary widely among different types and subtypes of individual LSDs most of them share central nervous system (CNS) involvement and lead to severe and progressing neurological symptoms such as mental retardation, dementia, blindness and motor and sensory disturbances. Also the peripheral nervous system, visceral organs, heart, skeletal muscles, bones and cartilages are affected to different extents.

LSDs are conventionally classified according to the chemical nature of the accumulating compound(s) which represents a disease-specific marker for many diseases. In this way, early

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Table 1. Localization and function of polypeptides deficient in lysosomal storage diseases and related disorders

Localization and Function	Protein	Corresponding Disease
Lysosome, soluble		
- hydrolases	**Mucopolysaccharidoses**	
	α-L-iduronidase	MPS I (Hurler, Scheie, Hurler-Scheie)
	iduronate 2-sulfate-sulfatase	MPS II (Hunter)
	heparan-N-sulfatase	MPS IIIA (Sanfilippo A)
	N-acetyl-α-glucosaminidase	MPS IIIB (Sanfilippo B)
	N-acetyl-glucosamine 6-sulfate-sulfatase	MPS IIID (Sanfilippo D)
	galactose 6-sulfate-sulfatase	MPS IVA (Morquio A)
	β-galactosidase	MPS IVB (Morquio B)
	N-acetylgalactosamine 4-sulfatase (ASB)	MPS VI (Maroteaux-Lamy)
	β-glucuronidase	MPS VII (Sly)
	hyaluronidase	MPS IX
	Sphingolipidoses	
	β-galactosidase	G_{M1} gangliosidosis
	β-hexosaminidase α-subunit	Tay-Sachs disease
	β-hexosaminidase β-subunit	Sandhoff disease
	β-galactocerebrosidase	globoid cell leukodystrophy (Krabbe)
	arylsulfatase A	metachromatic leukodystrophy
	α-galactosidase A	Fabry disease
	β-glucocerebrosidase (β-glucosidase)	Gaucher disease
	acid ceramidase	Farber disease
	acid sphingomyelinase	Niemann-Pick disease type A and B
	Glycoproteinoses	
	α-mannosidase	α-mannosidosis
	β-mannosidase	β-mannosidosis
	α-fucosidase	fucosidosis
	aspartylglucosaminidase	aspartylglucosaminuria
	α-N-acetylgalactosaminidase	Schindler disease
	Other	
	α-neuraminidase (α-sialidase)	mucolipidosis I (sialidosis I)
	α-1,4-glucosidase (acid maltase)	Pompe disease (glycogen storage disease II)
	acid lipase	Wolman disease, cholesteryl ester storage disease
	cathepsin K	pycnodysostosis
	palmitoyl protein thioesterase I	ceroid lipofuscinosis type 1
	tripeptidyl peptidase I	ceroid lipofuscinosis type 2
- cofactors	G_{M2} activator	G_{M2} gangliosidosis AB variant
	saposin B	metachromatic leukodystrophy
	saposin C	Gaucher disease
- other	protective protein/catA	galactosialidosis
	NPC2p	Niemann-Pick disease type C2

continued on next page

		Corresponding Disease
		mucolipidosis IV Danon disease
		infantile sialic acid storage disease, Salla disease cystinosis cobalamin F disease MPS IIIC (Sanfilippo C)
		ceroid lipofuscinosis type 3 (Batten disease) ceroid lipofuscinosis type 5
Endosome		
	NPC1p (lipid processing)	Niemann-Pick disease type C1
Golgi apparatus		
- phosphorylation	UDP-N-acetylglucosamine: lysosomal enzyme N- acetylglucosaminyl-1 phosphotransferase	mucolipidosis II (I cell disease) mucolipidosis III (pseudo Hurler polydystrophy)
Endoplasmic reticulum		
formylation	formylglycine generating enzyme	multiple sulfatase deficiency
- other	CLN6p CLN8p *(also ER-Golgi)*	ceroid lipofuscinosis type 6 ceroid lipofuscinosis type 8
Cytosol		
- vesicle formation and trafficking	HPS1-7	Hermansky-Pudlak syndrome type I-VII
	LYST (lysosomal trafficking regulator)	Chediak-Higashi syndrome

studies led to the recognition of mucopolysaccharidoses, sphingolipidoses and glycoproteinoses as distinct entities (Table 1). Disorders of these three families typically result from a deficiency of a soluble lysosomal hydrolase which fulfills an essential function in one of the catabolic cascades responsible for the breakdown of lysosomally delivered glycosaminoglycans (mucopolysaccharides), sphingolipids and glycoproteins, respectively. Hydrolase deficiencies account for approximately two third of the storage disorders known to date and oppose to LSDs that are due to deficiencies of polypeptides without hydrolytic activity. Examples are lysosomal, soluble cofactors essential for the activation of sphingolipids and lysosomal membrane proteins acting as ion channels or transporters. Other LSDs have been ascribed to deficiencies of polypeptides which are not localized to the lysosome but to upstream compartments. They can reside within the endosome, Golgi apparatus or the endoplasmic reticulum. The growing list of nonhydrolytic polypeptides

Table 2. Basic treatment concepts for lysosomal storage diseases (for details see text)

Therapeutic Strategy	Example of Clinical Application
Enzyme augmentation therapies	
Enzyme replacement therapy	
plasma exchange	only of historical interest (unsuccessful)
enzyme infusion	
M6P-dependent enzyme targeting	α-L-iduronidase for Fabry disease
mannose-dependent enzyme targeting	β-glucocerebrosidase for Gaucher type 1 disease
Transplantation therapy	
(a) for cross-correction of deficient recipient cells by therapeutic enzyme released from donor-type cells	
tissue/organ transplantation e.g., kidney, liver, spleen	only of historical interest (unsuccessful)
cell transplantation	
naive donor cells e.g., bone marrow stem cells	allogeneic bone marrow transplantation for MPS I
genetically modified cells (ex vivo gene therapy) e.g., bone marrow stem cells, fibroblasts, myoblasts	not clinically available (experimental stage)
(b) for substitution or repair of damaged recipient cells/tissues/organs by donor cells/tissues/organs	
tissue/organ transplantation e.g., cornea, kidney	kidney transplantation for Fabry disease
cell transplantation e.g., embryonic stem cells, astroglial progenitor cells	not clinically available (experimental stage)
In vivo gene therapy	not clinically available (experimental stage)
Enzyme enhancement therapy	
protease inhibitors	not clinically available
chemical chaperons	galactose for cardiac variant of Fabry disease
Substrate reduction therapy	
diet	low-cholesterol diet for cholesterol ester storage disease
inhibition of substrate synthesis	NB-DNJ for Gaucher disease
modification of substrate	cysteamine for cystinosis

involved in LSDs underscores that the normal lysosomal function requires more than a full complement of hydrolytic activities, and also depends on the integrity of various other factors inside and outside the lysosome. The extent of the network needed to maintain lysosomal homeostasis is illustrated by the LSD-related phenotypes of Hermansky-Pudlak and Chediak-Higashi syndrome, two diseases which result from defects of cytosolic polypeptides. The characterization of lysosomal and nonlysosomal polypeptides involved in LSDs has markedly increased our understanding of the lysosomal biology. It is also crucial for elaborating novel therapeutic strategies and for assessing the potential of established concepts for a given disease. Table 2 summarizes treatment strategies which will be discussed in this review.

Enzyme Replacement Therapy

Early experiments revealed that the metabolic defect of cultured fibroblasts from mucopolysaccharidosis patients can be compensated by addition of corrective factors which proved to be the wildtype counterparts of the deficient lysosomal enzymes.[2] The added enzymes are rapidly internalized into the lysosomal compartment where they catabolize the accumulated substrates. Importantly, only 1-5% of the normal cellular activity was required for correction. The detection of this corrective mechanism led to the optimistic prediction that LSDs should be generally treatable by administration of the respective intact lysosomal enzyme, a treatment strategy designated as enzyme replacement therapy (ERT).

The uptake of lysosomal enzymes into the lysosomal compartment of fibroblasts and other cells depends on receptor-mediated endocytosis via a mannose 6-phosphate receptor (MPR) (for a review see Chapter 2 in this book and refs. 3-5). The receptor binds mannose 6-phosphate (M6P) residues which are synthesized at N-linked carbohydrates of soluble lysosomal hydrolases during their biosynthesis and which play an important role also in the intracellular routing of newly synthesized lysosomal enzymes from the Golgi apparatus to the lysosome (biosynthetic pathway). After binding at the cell surface the receptor-ligand complexes cycle from the plasma membrane to an endosomal compartment where the ligands dissociate and reach the lysosome (endocytic pathway). Some cell types accomplish sorting of exogenous lysosomal polypeptides via additional carbohydrate recognition systems which bind e.g., terminal galactose (hepatocytes) or mannose residues (reticuloendothelial cells)[6] and for the saposin precursor also a carbohydrate-independent endocytic pathway has been described.[7] Due to the requirement of soluble ligands and their receptor-mediated routing to the endosomal/lysosomal compartment, ERT is a therapeutic option for deficiencies of soluble lysosomal polypeptides, but generally not applicable to LSDs caused by the lack of membrane-bound polypeptides and soluble polypeptides residing in upstream compartments of the endosome (Table 1).

ERT of animal models for various LSDs such as MPS I,[8] MPS IIIB,[9] MPS VI,[10,11] MPS VII,[12] Fabry disease,[13] Niemann-Pick disease[14] and Pompe disease[15] revealed that intravenously infused lysosomal enzymes are rapidly internalized by liver, spleen and other peripheral tissues, but usually do not enter the brain parenchyma in therapeutically efficient amounts. As a consequence the visceral, but not the CNS pathology can be improved.

Several efforts were undertaken to alter the biodistribution of infused enzymes, particularly to favour the delivery to the CNS. Studies in a mouse model of MPS VII revealed that recombinant β-glucuronidase can reach the brain parenchyma when it is injected into newborns whose blood-brain barrier (BBB) is still leaky.[16] Two weeks later, however, the BBB is fully differentiated and prevents further uptake of enzyme from the circulation. Experimental attempts to overcome the closed BBB comprise invasive strategies, e.g., intracerebroventricular infusion or temporary disruption of the tight junctions between cerebral endothelial cells by infusing hypertonic solutions (for a review see ref. 17). The associated risks of infection and neuropathological side effects directed attention to noninvasive delivery strategies based on conjugates between blood-brain shuttle vectors and therapeutic enzymes. Examples for shuttle vectors that have been investigated are certain antibodies which are transcytosed across the BBB and atoxic fragments of tetanus and cholera toxin which gain access to the CNS via retroaxonal transport.[17-19] The Tat peptide, a recently identified vector consisting of only 11 amino acids from the human immunodeficiency virus TAT protein[20] favours brain uptake of the 120 kDa protein β-galactosidase following intravenous infusion of a Tat-β-galactosidase fusion protein into adult mice.[21] Tat-tagged β-glucuronidase, however, was not transported to the brain parenchyma.[22] Though shuttle vectors have been used with some success to target conjugated enzymes to neurons and the brain, it is not yet decided how far the transportation rate is sufficient to achieve therapeutically efficient enzyme levels in the CNS.

The first "proof-of-principle" for the therapeutic efficacy of ERT in humans has been provided by the successful treatment of patients suffering from type 1 (nonneuronopathic) Gaucher disease.[23] The clinically available therapy makes use of mannose-terminated glucocerebrosidase

which is generated by removal of terminal carbohydrates from the N-linked oligosaccharides by sequential exoglycosidase treatment resulting in the exposure of the core mannose residues. This modification targets the enzyme efficiently to cells of the monocyte-macrophage lineage which are the main site of storage and mannose receptor expression. Treatment prevents progressive manifestations of the disease and ameliorates disease-associated anemia, thrombocytopenia, organomegaly, bone pain and bone crises.[24]

Owing to the potential of ERT to improve the visceral pathology in animal models and human Gaucher disease, clinical trials of ERT have been initiated for Fabry disease,[25-28] Pompe disease,[29,30] MPS I,[31,32] MPS II [33] and MPS VI (not yet reported).

Classical Fabry disease is characterized by the accumulation of the glycosphingolipid globotriaosylceramide leading to neuropathic pain and death in early adulthood due to renal failure, cardiovascular disease or cerebrovascular complications.[25] Infusion of M6P-bearing recombinant human α-galactosidase A (α-gal A) diminishes globotriaosylceramide levels in plasma and tissues, decreases pain, stabilizes the kidney function, partially normalizes the hypertrophic heart mass, reverses hearing deterioration and improves peripheral nerve and sweat function.[26-28] ERT in Pompe disease and MPS I has been similarly promising.[29-32] Thus, intravenous administration of recombinant human α-glucosidase can improve muscle morphology and function in classic infantile Pompe disease when treatment is started before irreversible damage has occurred.[30] Recombinant human α-L-iduronidase has been recently approved in the European Union and the United States and MPS I patients have begun to receive therapy in May 2003.

Preclinical and clinical studies demonstrate that ERT can reduce years of substrate accumulation in visceral tissues leading to improvement of pathological aspects and gain of life quality. ERT seems to have, however, no effect on the CNS manifestation of neuronopathic LSDs and ameliorates bone and cartilage problems only to a limited extent. Other problems are immune responses to the replacement protein,[34] the need for life-long intervention and the high costs of treatment (e.g., US $160,000 per year for a 70-kg Fabry disease patient; US $100,000 to US $750,000 per year for a typical adult with Gaucher disease, depending on the enzyme dosage).

Transplantation Therapy

Studies on the biosynthetic targeting pathway of lysosomal enzymes revealed that a substantial fraction of newly synthesized soluble lysosomal enzymes is not targeted to the lysosome but released from the cell.[3-5,35] Numerous feeding and coculture experiments demonstrated that target cells can internalize hydrolases released by producer cells in a M6P-dependent manner. Arylsulfatase A (ASA), for example, is efficiently endocytosed from secretions of ASA-overexpressing baby hamster kidney cells and the uptake can be completely inhibited by M6P.[36] Endocytosis restores the capacity of ASA-deficient cells to hydrolyse the sphingolipid sulfatide, which accumulates if ASA is lacking.[37] The metabolic correction of an enzyme-deficient cell by the transfer of the therapeutic enzyme from a enzyme-competent cell is designated as cross-correction.[38] It is usually due to the described carbohydrate-dependent endocytosis pathways, however, another transfer mechanism that requires direct cell-to-cell contact exists.[39] Cross-correction allowed the extension of the basic concept for ERT: a supply of deficient cells does not necessarily depend on the administration of the therapeutic enzyme itself, but should be also achieved by transplantion of enzyme-producing cells which transfer the enzyme by a release/uptake mechanism or direct cell-to-cell transfer to neighbouring cells.

Transplantation therapy has been applied to animal models and patients in many variations. Early attempts to attain systemic cross-correction by transplantation of whole organs or amnion cells were unsuccessful[40] and were followed by hematopoietic stem cell transplantation using naive stem cells from bone marrow or peripheral blood.

Bone Marrow Transplantation

Kinetic studies in mice revealed that 30% of microglial cells are of donor-type 12 months after bone marrow transplantation (BMT).[41-43] The repopulation of the CNS with bone

marrow-derived microglial cells suggested a convenient route of transferring therapeutic enzyme into the brain parenchyma. BMT was therefore proposed to solve the main limitation of ERT, namely the inpermeability of the BBB for lysosomal polypeptides. This notion was supported by BMT therapy of a cat model of α-mannosidosis, which led to the appearance of α-mannosidase in neurons and other cells of the CNS concomitant with the loss of intracellular storage vacuoles.[44] Treated cats showed little or no progression of neurologic signs 1-2 years post transplant, whereas untreated cats became severely impaired and reached end-stage disease by 6 months of age. This study greatly pushed the interest in BMT therapy for various LSDs with CNS involvement. However, further BMT trials in animal models were less effective in most cases and no improvement of the brain pathology was detectable e.g., in a cat model of G_{M2} gangliosidosis treated by an identical protocol.[45]

The variability of therapeutic success has been ascribed to various factors, including the different amounts of individual lysosomal enzymes in secretions of enzyme-producing cells.[35] It has been shown recently that bone marrow-derived cells, in contrast to transfected hamster cells for example, deliver mainly unphosphorylated and thus uptake-incompetent ASA and cathepsin D.[36] Thus, cell type- and may be enzyme-specific variations in the extent of mannose phosphorylation might also contribute to the variable results. The catabolic defects of cells from Tay-Sachs patients or a mouse model of types A and B Niemann-Pick disease can not be corrected by feeding with the respective therapeutic enzyme due to the failure of the enzymes to reach the lysosomal compartment.[46,47] It was hypothesized that the lipid accumulation in these two sphingolipidoses interferes with the correct trafficking of lysosomal enzymes. Primary kidney cells from a mouse model of the sphingolipidosis metachromatic leukodystrophy (MLD), however, readily internalize the deficient enzyme ASA leading to the decline of accumulated sulfatide.[48] These findings demonstrate substantial differences in the cellular defects even within one group of related LSDs necessitating different treatment strategies.

Since 1980 patients for nearly 20 LSDs have been treated by BMT. Most transplantations have been done for mucopolysaccharidoses, and within this group for MPS I. Long-term follow-up studies of treated MPS I patients indicated that BMT can ameliorate visceral symptoms, such as hepatosplenomegaly, respiratory problems and cardiac function and might arrest or slow down neurological deterioration.[49,50] However, bone, cartilage and heart valves do not respond to treatment. Currently, BMT for MPS I is recommended only for the mild Hurler variant and before the second year of age. Less encouraging results have been obtained for other mucopolysaccharidoses such as MPS II, MPS III and MPS IV, lipidoses such as Farber disease and the glycoproteinosis aspartylglucosaminuria.[51-53] On the other hand, amelioration of visceral symptoms, bone disease, stabilization of neurocognitive functions or even improvement of psychomotor development has been reported for patients suffering from the glycoproteinosis α-mannosidosis or fucosidosis.[54,55] The data collected by multiple transplantation centers support the conclusion of animal studies that the outcome of BMT largely depends on the type and the stage of the disease. While BMT, especially when performed early, can have beneficial effects on visceral symptoms and to a lesser extent on bone disease, the effect on neurological symptoms varies. However, BMT generally does not result in a normal phenotype and is associated with a mortality rate of 10% even if an HLA-identical donor is available.[56,57] Therefore, the risk and possible benefit of BMT have to be balanced carefully for each individual patient.

Ex Vivo Gene Therapy

With the cloning of the cDNAs for polypeptides deficient in LSDs and the development of appropriate vector and vector packaging systems donor cells could be genetically modified by ex vivo gene therapy prior to transplantation. The rationale for this approach is the elevation of the enzyme production and delivery by constitutive expression of the correcting enzyme from a strong, usually viral promoter. Furthermore, ex vivo gene therapy allows the use of the patient's own cells as enzyme-producers thereby eliminating the risk of immune responses to unmatched donor cells or graft-versus-host disease. Various cell culture experiments verify that enzymes

released by cells which have been transduced with vectors based on retrovirus, adenovirus, adeno-associated virus, herpes simplex virus and others are correctly processed and can be taken up by target cells.[58]

Bone Marrow Stem Cell Gene Therapy

Of the various gene therapy approaches under investigation, hematopoietic stem cell-mediated gene therapy has attracted the most interest and its therapeutic potential has been analysed in a variety of animal models. The therapeutic potentials of conventional allogeneic BMT and bone marrow stem cell gene therapy have been compared in a mouse model of MPS I.[59] Transplantation of unmodified wildtype bone marrow was effective in reducing storage in liver and spleen, but not in kidney or brain. Gene therapy using bone marrow overexpressing human α-L-iduronidase from a retroviral vector, however, also corrected the pathology of kidney, choroid plexus, and thalamus. This study clearly supports the notion that bone marrow stem cell gene therapy can be superior to conventional BMT.

Transplantation of MPS VII mice with syngeneic bone marrow cells overexpressing β-glucuronidase from a retroviral vector resulted in resolution of storage in liver and spleen, but had no effect on brain and bone pathology.[60] A comparable response was detectable in a mouse model of MLD transplanted with bone marrow cells overexpressing ASA from a murine stem cell virus-based vector.[61] Sulfatide storage could be reduced in kidney and liver, but not in the CNS, though up to 30% of normal enzyme levels were present in the brain and some behavioral improvement was detectable.[62] On the other hand, bone marrow stem cell gene therapy using the same vector system mediating protective protein/cathepsin A overexpression was associated with significant amelioration of the CNS pathology in a mouse model of galactosialidosis.[63] These examples point to the potential of retrovirus-mediated gene transfer to bone marrow cells for improving systemic disease in mice, but similar to allogeneic BMT this procedure in treating CNS pathology is highly variable.

Our group attempted to improve the therapeutic potential of bone marrow stem cell gene therapy by increasing the ratio between the amount of extracellularly delivered and intracellularly retained enzyme. Enhanced misrouting of an overexpressed lysosomal enzyme from the Golgi apparatus to the extracellular space is expected to be advantageous since more enzymatic activity is available for being taken up by deficient recipient cells. An ASA mutant which lacks the M6P-bearing N-linked carbohydrates, but retains full activity and stability, is delivered from retrovirally transduced cells to a much higher extent than the wildtype enzyme because it escapes from binding to the MPRs in the trans Golgi network and thus follows the secretory route.[64] Transfer of the mutant ASA cDNA into ASA knockout mice by bone marrow stem cell gene therapy resulted in a twofold increase of the ASA serum level compared to mice transplanted with bone marrow expressing the wildtype ASA from the same retroviral vector. The elevated level of extracellular enzyme, however, did not improve the therapeutic potential of treatment, supporting findings by others[65] that M6P residues are crucial for the efficient targeting of therapeutic enzyme to affected cells in certain LSDs. In order to induce hypersecretion of phosphorylated lysosomal enzymes MPR-specific ribozymes have been recently constructed. The ribozymes deplete MPRs in cultured cells by hydrolysis of the receptor mRNAs and thereby mediate hypersecretion of ASA expressed from a cotransfected ASA cDNA.[66] Future experiments will determine if MPR-specific ribozymes improve conventional gene therapy approaches by increasing enzyme delivery from genetically modified donor cells.

In larger animals including man retrovirus-based gene therapy is associated with particular problems. Thus, autologous bone marrow from dog models of fucosidosis and MPS I which was transduced with retroviral vectors encoding α-fucosidase and α-L-iduronidase, respectively, failed to engraft after transplantation.[67,68] The authors supply evidence that the nonmyeloablated recipients developed a cellular immune response which specifically eliminated transgene-expressing donor-type cells. Unstable engraftment as well as low transduction efficiency was also noticed in a clinical trial analysing the fate of retrovirally transduced

autologous CD34+ cells after transplantation into Gaucher patients.[69] Due to these complications, the bases of which are not fully understood, bone marrow stem cell gene therapy has not been successfully applied to larger animals so far.

Other ex Vivo Gene Therapy Approaches and Intracerebral Grafting

Beside hematopoietic stem cells, a variety of other cell types has been transplanted including neuronal and mesenchymal stem cells and differentiated cells such as fibroblasts, myoblasts and others. Most transplantation studies were undertaken in a mouse model of MPS VII which lacks the soluble lysosomal enzyme β-glucuronidase. The enzyme deficiency leads to the accumulation of partially degraded chondroitin, dermatan, and heparan sulfates in peripheral tissues and brain and the development of most of the symptoms exhibited by human MPS VII patients, including progressive visual and cognitive deficits and skeletal deformities.[70] The intraperitoneal implantation of collagen lattices which bear autologous fibroblasts expressing β-glucuronidase from a retroviral vector results in the disappearance of storage in liver and spleen and was therefore proposed to be a treatment option for somatic manifestations of LSDs.[71,72] Also transplantation of retrovirally transduced myoblasts into skeletal muscle induces a disappearance of lysosomal storage lesions in the liver and spleen, but not in the brain of MPS VII mice.[73] To overcome the BBB, cells have been grafted directly into the brain. Embryonic stem cells and neural or oligodendroglial progenitor cells offer the combined possibility of delivering enzyme-competent cells to the CNS as well as supplying descendents which might substitute recipient cells which have been lost during the course of the disease. Immortalized murine or human neural progenitor cells injected into cerebral ventricles of newborn MPS VII spread over the brain, integrate into the CNS architecture and transmit β-glucuronidase to recipient cells resulting in a widespread clearance of storage in neurons and glial cells.[74,75] Clearance was also observed following intracerebral grafting of amnion cells or fibroblasts which overexpress the enzyme from an adenoviral and retroviral vector, respectively.[76,77] Unlike neural progenitor cells and amnion cells transplanted fibroblasts did not migrate away from the injection site and the therapeutic effect was locally restricted to the vicinity of the grafts. Additionally, the correction was only transient, possibly due to the loss of the transplanted cells. To avoid graft rejection enzyme-producing cells have been enclosed in biocompatible, immuno-isolating devices, such as alginate microcapsules (for a review see ref. 78). Introduction of microencapsulated β-glucuronidase-expressing cells into the brain ventricles of MPS VII mice demonstrated biochemical, histological and behavioral improvement.[79] Although the available systems are not yet adapted to larger animals[80] microcapsules might develop into valuable tools for the treatment of CNS diseases.

In Vivo Gene Therapy

Vector systems, which (different from conventional retroviral vectors) can infect also nondividing cells, offer the possibility to transduce differentiated cells in vivo without the need of explantation, in vitro cultivation/stimulation and subsequent implantation (for a review see ref. 58). In vivo gene transfer should also allow the direct and endocytosis-independent correction of affected cells. In contrast to ERT and transplantation therapy, in vivo gene therapy is therefore a theoretical option to supply deficient cells with polypeptides which can not reach their site of function by receptor-mediated endocytosis, because they lack the signals for internalization or reside apart from the endosomal/lysosomal compartment. Significant progress has been made in the improvement of adenovirus, adeno-associated virus and retrovirus vectors as gene transfer vehicles into living organisms.

The therapeutic potential of in vivo gene transfer by adenoviral vectors has been determined in a variety of animal models, the most comprehensive data being available for MPS VII mice. Systemic injection of adenovirus vectors mediating overexpression of human β-glucuronidase resulted in pathological improvements in multiple visceral organs of adult MPS VII mice, but no effect on the CNS pathology, skeletal deformities and eye disease was observed.[81-83] A single intravenous injection of the vector into neonatal mice, however, also

prevented/reduced lysosomal storage in brain, cornea and retina and corrected the bone and cartilage disease.[83,84] Perinatal treatment thus allows transfer of vector and/or recombinant enzyme to tissues which are not accessible in the adult mouse. As a possible alternative to early treatment, vectors have been injected directly into the brain of adult MPS VII mice. Stereotactic injection into the striatum resulted in focal, intense β-glucuronidase expression near the injection site and transfer of enzyme to the corpus callosum, ventricles and neocortex.[85] This study revealed a marked reduction in neuronal and glial storage throughout most of the treated hemisphere, but also the untreated hemisphere could be partially corrected indicating the cross-correction of brain cells by enzyme which is capable to reach the contralateral hemisphere by diffusion.

Similar encouraging results have been obtained with adeno-associated virus (AAV) vectors. Different from adenoviral vectors, AAV vectors integrate into the host genome resulting in a more stable transgene expression. Low immunogenicity and lack of inflammatory side effects are further advantages of this vector class. First, adult MPS VII mice were injected intravenously with a recombinant AAV vector encoding the human β-glucuronidase.[86] Although the vector produced therapeutic enzyme levels in the liver, enzyme delivery to the brain was inadequate. Newborn mice, however, developed therapeutic levels also in brain and retina by 1 week after intravenous vector administration.[87,88] Expression persisted for at least 1 year at levels sufficient to reduce/prevent lysosomal storage in all tissues including neurons, microglia and meninges. Survival and hearing abilities were significantly improved and bone length, body weight, and retinal function were maintained at near normal levels throughout the life of the mice. Intracerebral administration of the vector to newborn or adult mice resulted in near normal enzyme activities in total brain and the elimination of storage granules in brain tissue.[86,89-91] An additional improvement of cognitive functions was achieved when the mice were treated as newborns.[91] Surprisingly, injection of vector into the vitreous humor of the eye of adult mice also led to a substantial clearance of lysosomal storage in the brain.[92] Regions which received direct visual input, such as the tectum responded more clearly than nonvisual regions adjacent to it. The authors supplied evidence that enzyme, rather than the vector, disseminated within the brain by both neuronal transport and diffusion. Axonal transport from the retina might thus be exploited to deliver therapeutic enzymes into the brain providing a less invasive delivery method than intracerebral gene delivery.

The last main class of vectors which is currently tested for in vivo gene transfer applications comprises lentiviral and nonlentiviral retrovirus vectors. Both subclasses stably integrate into the nuclear host genome. Unlike the lentiviral vectors, which bear a nuclear targeting signal transporting the viral genome through nuclear membrane pores to the nucleoplasma, the nonlentiviral retroviruses lack the signal and can therefore only integrate during mitosis when the nuclear membrane is dissolved. The requirement of cell division makes nonlentiviral vectors unattractive candidates for in vivo gene transfer. Despite this limitation, the first successful prevention of a LSD in a large animal by gene therapy was achieved with a nonlentiviral vector.[93] In this approach newborn MPS VII dogs were intravenously injected with a Moloney murine leukemia virus (MLV)-based retroviral vector harboring the canine β-glucuronidase cDNA driven by the human α1-antitrypsin promoter. Treatment resulted in stable serum levels of the enzyme, near to normal body weights, improvement in bone and joint abnormalities, and lack of corneal clouding, cardiovascular disease and neurological symptoms. This study demonstrates that the rapid liver growth in the perinatal period allows transduction of 3% of hepatocytes by a MLV-based vector resulting in sustained expression of a therapeutic protein from the liver for at least 17 months. A comparable study in the mouse model for MPS VII revealed that enzyme secreted by neonatally transduced hepatocytes was taken up by other tissues, as the average enzyme activity was >13% of normal in somatic organs and 2% of normal in brain.[94]

The outcomes of experiments with lentiviral vectors were similar to those with AAV vectors. Thus, a human immunodeficiency virus (HIV)-based lentiviral vector encoding β-glucuronidase administered to the brain of young adult MPS VII mice by multiple injections induced correction of the pathology throughout the brain, suggesting regression of advanced

brain lesions in the entire CNS.[95] By injection into the eye, a feline immunodeficiency virus (FIV)-based vector has been successfully used to correct the cellular pathology in the cornea, iris, and retina of MPS VII mice.[96] Injection into the striatum resulted in the reversal of the brain pathology in both hemispheres and the restoration of established impairments in spatial learning and memory.[97]

In view of the remarkable success obtained with in vivo gene transfer to the CNS it is important to emphasize that the presently available vector systems bear considerable and possibly still unrecognized risks. One issue which has been poorly addressed is the possibility that a massive overexpression of a lysosomal enzyme might have adverse side effects on the transduced cells. This notion is supported by the reduced activity of various lysosomal sulfatases in MPS VI fibroblasts expressing 50-fold more ASB than wildtype fibroblasts.[98] It was speculated that overexpression of ASB may deplete the formylglycine generating enzyme (FGE) in the ER which is essential for the activation of lysosomal sulfatases. High-level overexpression can also reduce the intracellular activity of lysosomal enzymes distinct from sulfatases suggesting the misrouting of newly synthesized lysosomal enzymes to the plasma membrane due to the over-saturation of MPRs in the trans Golgi network.[99] Long-term in vivo studies are mandatory to substantiate that enzyme overproduction e.g., in neuronal cells does not induce a new cellular and pathological phenotype which outweighs beneficial effects of enzyme-mediated metabolic correction.

The uncontrollability of the proviral integration which can induce tumorigenesis by disruption of tumor suppressor genes or activation of proto-oncogenes is another safety concern for gene therapy with integrating vectors. Recently, high frequency of tumor development has been found in a β-glucuronidase transgenic mouse strain implicating an integration site-dependent oncogenic potential of the used gene transfer vector.[100] Also AAV vectors and retroviral vectors used in preclinical and clinical trials induced malignant transformation.[101,102] The presently unsolved safety problems are fundamental drawbacks which retard the clinical implementation of gene therapy for LSDs and other diseases.[103]

Enzyme Enhancement Therapy

In contrast to ERT, transplantation therapy and gene therapy, enzyme enhancement therapy (EET) exploits the residual activity of the mutant endogenous polypeptide (for review see ref. 104). Residual enzyme activities are present in mild subtypes of most LSDs where e.g., a missense mutation apart from the active center causes thermodynamic instability of the polypeptide. As a consequence only a few molecules adopt proper folding. The majority is misfolded and rapidly degraded either through the ubiquitin-proteasome pathway, controlling the export of proteins from the ER or through lysosomal proteases.[105] In cell culture experiments two strategies of enzyme activation have been successfully applied. The first strategy utilizes protease inhibitors which may increase the half life of misfolded, but lysosomally targeted enzymes by reducing their proteolytic degradation rate. Thus, the activity of ASA and the degradation of sulfatides can be partially restored in fibroblasts from patients suffering from late-onset forms of MLD by addition of inhibitors of cysteine or thiol proteinases to the medium.[106,107] However, due to the expected profound side effects the therapeutic potential of proteinase inhibitors has not been elucidated so far.

The second strategy utilizes so-called chemical chaperones, specific small-molecule ligands which bind to the catalytic site of an enzyme and rescue mutant polypeptides by assisting their correct folding in a prelysosomal compartment.[108] It has been proposed that such compounds stabilize a folding intermediate whose concentration is rate limiting for subsequent folding steps, eventually leading to the adoption of the correct tertiary structure. As a consequence of stabilization, a higher percentage of mutant enzyme is properly folded, passes the quality control system of the ER and is targeted to the lysosome where its conformation might be stabilized by the acidic conditions. Cell culture experiments demonstrated that 1-deoxygalactonojirimycin (DGJ), a potent competitive inhibitor of α-galactosidase A (α-gal A), increases the activity of α-gal A in lymphoblasts from late-onset Fabry patients up to 8-fold at subinhibitory intracellular concentrations.[109,110] Consistent with

a role of DGJ in assisting proper folding, this increase is accompanied by a redistribution of the mutant enzyme from prelysosomal compartments to the lysosome. Also galactose and melibiose increase the activity and amount of a variety of mutant α-gal A polypeptides.[111,112]

The first clinical evidence for the feasibility of EET in a human LSD has been supplied by the successful treatment of a 55-year-old man suffering from a late-onset cardiac variant of Fabry disease with 2.4% of normal α-gal A activity.[113] Daily intravenous infusion of 1g galactose per kg body weight improved cardiac contractility and reduced cardiac mass within three months. Because of the substantial improvement of the end-stage hypertrophic cardiomyopathy cardiac transplantation was no longer required. More recently, a galactose-derivative inhibiting lysosomal β-galactosidase, the deficiency of which causes G_{M1} gangliosidosis or Morquio B disease, has been investigated.[114] The compound restored mutant enzyme activity in cultured human or murine fibroblasts at subinhibitory intracellular concentrations, resulting in a decline of intracellular substrate storage. Furthermore, the inhibitor significantly enhanced the enzyme activity in the brain and other tissues of a mouse model of juvenile G_{M1} gangliosidosis. Chemical chaperons may therefore be useful for certain patients with mild LSDs with or without CNS involvement.

Substrate Reduction Therapy

The therapeutic strategies which have been described so far try to elevate the activity of a polypeptide whose activity is below the critical threshold required to maintain the normal lysosomal function. ERT, transplantation therapy (with or without ex vivo gene therapy), in vivo gene therapy and EET can therefore be classified as enzyme augmentation therapies. A second class of therapy approaches, designated as substrate reduction therapies (SRT), aims to counteract storage by nonenzymatic means. Presently, inhibitors of anabolic enzymes involved in the synthesis of compounds which are stored in glycosphingolipidoses are investigated (for review see ref. 115). The rationale for the use of inhibitors is to reduce the de novo synthesis rate of an accumulating compound to a level where the residual activity of the mutant catabolic enzyme is sufficient to prevent pathological storage. Similar to EET, SRT therefore depends on a residual activity of the mutated enzyme. The glucose analogue N-butyldeoxynojirimycin (NB-DNJ) blocks the glucosylceramide synthase which catalyzes the synthesis of glucocerebroside (glucosylceramide) by transferring a glucosyl moiety onto ceramide.[115] Cell culture experiments demonstrated that NB-DNJ can reduce the synthetic rate of glycosphingolipids and diminish storage of these compounds in patient's cells without causing toxicity.[116] Inhibitors of the glucosylceramide synthase were therefore proposed to be of possible benefit for mild variants of all LSDs which involve storage of glucosylceramide-based glycosphingolipids, including Gaucher disease, Fabry disease, G_{M1} gangliosidoses and G_{M2} gangliosidosis (Tay-Sachs and Sandhoff disease). Another compound, L-cycloserine, an inhibitor of the first enzyme of the sphingolipid pathway, 3-ketodihydrosphingosine synthetase, also diminishes the synthetic rate of galactosylceramide-based sphingolipids and might thus be of possible benefit for the treatment of further sphingolipidoses such as Krabbe disease.[117,118]

To date the majority of studies evaluating SRT have utilized NB-DNJ. Oral administration of this compound to wildtype mice revealed that visceral glycosphingolipids can be depleted in a dose-dependent manner by up to 70% without affecting survival.[119] In a mouse model of the G_{M2} gangliosidosis Tay-Sachs disease a 50% reduction of cerebral G_{M2} ganglioside storage was detected indicating that NB-DNJ is able to cross the BBB in an amount sufficient to prevent substrate accumulation.[120] Also a mouse model of the G_{M2} gangliosidosis Sandhoff disease showed reduction of ganglioside storage in the CNS.[121] In addition, the treated mice have delayed symptom onset and increased life expectancy. Interestingly, a combination between BMT and SRT further prolonged survival pointing to a synergistic effect between the two treatment strategies.[122] Due to the good response of knockout mice combination therapy might therefore be especially advantageous for early-onset forms of the disease characterized by a very low or absent residual enzyme activity.[115]

To assess the potential of NB-DNJ (OGT 918) in reducing visceral glycosphingolipid storage in humans a clinical trial in type 1 Gaucher disease was initiated.[123] Treatment with 100 mg oral OGT 918 three times daily resulted in a reduction of the enlarged spleen and liver volumes, slight improvement of haematological parameters and a decrease in the number of Gaucher macrophages in bone marrow. Adverse side effects of long-term treatment included weight loss, tremor, peripheral neuropathy and gastrointestinal complaints such as chronic diarrhoea.[124] Studies in mice additionally indicated that NB-DNJ can inhibit liver glycogen breakdown, induce partial lymphoid organ shrinkage and cause malformation of spermatozoa resulting in infertility.[125-127] To reduce or abrogate side effects which can be ascribed to the inhibition of unspecific enzymes, glucosylceramide synthase inhibitors with increased specificity are under investigation. Thus, the ceramide analogue D-threo-1-ethylendioxyphenyl-2-palmitoylamino-3-pyrrolidino-propanol diminishes renal and hepatic globotriaosylceramide storage in a mouse model of Fabry disease without inducing weight loss and acellularity of lymphatic organs.[128]

A different substrate reduction strategy has been realized almost 20 years ago for the LSD cystinosis, a disease which is due to the functional deficiency of a lysosomal membrane protein exporting the disulfide amino acid cystine from the lysosomal compartment into the cytosol. The defect causes the accumulation of cystine and a disease characterized by renal dysfunction, growth retardation, photophobia and other symptoms (for a review see ref. 129). Cystinosis fibroblasts which were grown in the presence of 0.1 mM cysteamine, a low-molecular weight compound bearing a sulfhydryl and an amino group, showed a decline of storage by 90% within 1 hour.[130] Eventually it was demonstrated that cysteamine reacts with cystine by a sulfhydryl-disulfide exchange to the mixed disulfide cysteamine-cysteine (and cysteine) which can exit from the lysosome through an intact lysine transporter.[131] In this elegant approach storage can be abrogated by a covalent modification of the accumulating compound bypassing the lysosomal defect. Cysteamine is routinely used to treat cystinosis patients and retards renal deterioration, improves growth and dissolves corneal cystine crystals.[129]

Conclusions

Research on LSDs led to enormous progress in the past 10 years. Knockout mouse models which have been created for virtually all LSDs contributed substantially to this progress and provided new insights into the pathogenesis and therapeutic possibilities. The development of eukaryotic expression systems which allow for the large-scale production of recombinant human enzymes rendered the transfer of ERT from the laboratory to the clinic possible. ERT is now clinically available for Gaucher disease type 1, MPS I and Fabry disease and under development for several other LSDs which primarily affect visceral tissues. The treatment of the CNS remains complicated, but new promising therapy concepts have been elaborated. Although still at an experimental stage, EET-based strategies might be of future use for mild variants of certain LSDs. Novel inhibitors for anabolic enzymes catalyzing the synthesis of sphingolipids directed new attention to the old concept of SRT. First clinical data for Gaucher disease type 1 suggest that SRT might be a therapeutic option for patients who can not be treated by ERT. Ex vivo gene transfer into hematopoietic stem cells appears to be superior to conventional allogeneic bone marrow transplantation, but the transfer of the established protocols from mice to humans failed so far. Optimized vectors with increased efficacy and safety are required to overcome the present limitations. Remarkable advances have been made using adenovirus, adeno-associated virus and lentivirus vectors for gene transfer directly into the CNS of experimental animals. These vector systems mediate transgene expression levels sufficient to prevent the progression or even to reverse the CNS manifestations of certain LSDs in mice. However, many obstacles need to be resolved before any gene transfer protocol can be routinely applied to human patients. A main hurdle that must be overcome is the low safety of the available vector systems which can not be understated in view of recent problems.[102,132]

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CHAPTER 11

Lysosomal Proteome and Transcriptome

Jobst Landgrebe* and Torben Lübke

Analysis of Lysosomal Structure and Function Using Proteomics and Transcriptomics

As reflected in this monograph, a lot of knowledge about the lysosomal compartment has been acquired since the discovery of the organelle by de Duve and others in the 1950s. Nevertheless, a lot of questions are still open, and some of them can be addressed using screening techniques. Among them are questions about the structure of the lysosomal membrane, the function of its proteins, and the effect of lysosomal dysfunction on cell functions. Although the proteins of the lysosomal matrix are well known, little is realised about the proteins of the lysosomal membrane. In the first section of this chapter, we describe the most important proteome analysis related techniques used to purify and analyse lysosomal structures that can help to define the complete set of matrix proteins and to describe the proteins of the lysosomal membrane. In the second section, we delineate the gene expression profiling technique and briefly describe approaches which can be used to analyse the influence of lysosomal dysfunctions on the cell, as well as on the function of lysosomal proteins. Because transcriptome and proteome analyses of lysosomal structure and function are still only in the initial stage, we will focus on the description of the methods and the major aims.

Proteome Analysis of Lysosomal Proteins

The term "Proteome", coined in 1995 by Marc R. Wilkins, defines the protein complement of the genome (functional genomics) and claims to describe the entirety of all proteins existing of an organism, tissue, cell or organelle at a given time point under certain conditions.[1,2] The ideal proteome analysis approach reflects not only the numerous proteins, but also the quantitative equilibrium of all proteins and their modifications. To date, an ideal proteomic analysis of an organelle like the lysosome is far from reality since some sets of proteins are generally underestimated: Hydrophobic membrane proteins with several transmembrane spanning domains may be lost due to their amphiphilic nature and insufficient extraction methods. Moreover, very basic proteins, very small and very large proteins, as well as low abundant proteins often fail to be detected as a result of technical limitations in sample preparation, sample separation or detection methods.[3]

Today, approximately 50 lysosomal matrix proteins (see Chapter 6) and 20 lysosomal membrane proteins (see Chapter 4) are known. Whereas the vast majority of the mannose-6-phosphate residue tagged matrix proteins are thought to be identified and functionally characterised in detail (see Chapters 6, 7 and 8), the proteome of the lysosomal membrane is considered to be largely incomplete since the few known proteins described so far are not sufficient to fulfil the assigned tasks—in particular, transport processes.[4] Defective lysosomal membrane transport proteins were shown to be responsible for diseases like cystinosis,[5] Salla[6] or Niemann-Pick type C,[7] whereas the loss of structurally important lysosomal membrane proteins like LAMP-2[8] and LIMP-2[9] leads to

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Lysosomes, edited by Paul Saftig. ©2005 Eurekah.com and Springer Science+Business Media.

symptoms which do not directly resemble classical lysosomal storage diseases. This situation makes the search for new lysosomal proteins by proteomics necessary and important.

Sample Preparation for Lysosomal Proteomics

In order to elucidate the protein composition of a certain cellular compartment, it is important to first obtain highly purified sets of the appropriate organelle(s). For the preparation of pure lysosomal matrix proteins, one can easily exploit the existence of mannose-6-phosphate residues (see Chapter 2). The preparation of lysosomal membrane proteins needs more sophisticated protocols (see below).

Sample Preparation for Lysosomal Matrix Proteins

Generally, lysosomal matrix proteins are specifically targeted through the mannose-6-phosphate (M-6-P) residue to lysosomes. Journet et al[10,11] took advantage of this specific covalent modification in two attempts, by treating cultured human U937 cells with ammonium chloride (NH_4Cl) which interferes with the regular transport of lysosomal matrix proteins and results in an increased secretion of M-6-P tagged proteins.[12] Thereafter, M-6-P proteins containing supernatants were collected, precipitated, dialysed and finally affinity-purified on a cation-independent M-6-P receptor ($MPR300^{CI}$) column, as described by Hoflack et al.[13] By extensively washing of the column, unspecifically bound proteins were removed. Specifically bound M-6-P proteins were competitively eluted with mannose-6-phosphate, concentrated and applied to either 1D or 2D gel electrophoresis, followed by mass spectrometry analysis.[10,11]

Another approach takes advantage of a mouse embryonic fibroblast cell line deficient in both M-6-P receptors, namely cation-independent $MPR300^{CI}$ and cation-dependent $MPR46^{CD}$.[14] This receptor-deficient cell line constitutively releases M-6-P proteins into the medium, from where they can easily be affinity-purified and analysed as described above (unpublished data). For both methods it is advisable to decrease the amount of fetal calf serum in the medium in order to elevate the ratio of secreted lysosomal proteins to calf serum proteins.

Sample Preparation for Lysosomal Membrane Proteins

To date, no real systematic proteome analysis of lysosomal membrane proteins has been published—illustrating the difficulty of this task. There is no convenient way to enrich or purify lysosomal membranes or lysosomal membrane proteins, as described for lysosomal matrix proteins. Due to the lack of a unique sorting signal for lysosomal membrane proteins (Chapter 4), one has to isolate pure lysosomes from tissue or cell culture by conventional biochemical methods. In the last decades, many isolation procedures have been developed, but none of those methods has been validated by systematic protein identification methods.

In 1970 Stahn et al,[15] and in 1980 Harms et al,[16] published protocols to isolate lysosomes from rat liver and human skin fibroblasts, respectively, by carrier-free electrophoresis, also named free-flow electrophoresis (FFE). This method is based on differences in electrokinetic properties between lysosomes and nonlysosomal structures. The high glycosylation status of many lysosomal proteins, accompanied by numerous negatively charged sialic acid residues, leads to the separation of lysosomes from other organelles in an electric field where the crude mixture of organelles flows through. However, the most critical step of this procedure is the preparation of the crude organelle mixture applied to the FFE apparatus by differential centrifugation, as inappropriate samples tend to aggregate and result in incomplete separation.[16] FFE was shown to allow in-depth proteome analysis of mitochondria from *Saccharomyces cerevisiae*[17] and, by a novel approach called immune free-flow electrophoresis (IFFE) was applied to peroxisomes from mouse liver.[18] Unfortunately, expensive technical equipment and the lack of skilled staff required for sample preparation and separation prevents the wider propagation of FFE.

Another inventive method for isolating lysosomes from fibroblasts has been described by Diettrich et al.[19] The authors used endocytosis of superparamagnetic colloidal iron dextran (FeDex), followed by the separation of the iron-containing lysosomes in a strong magnetic field, resulting in a 40-fold enrichment in lysosomal marker proteins.

Nevertheless, the most common strategy for purifying lysosomes from crude tissue or cell homogenates links differential centrifugation steps to isopycnic centrifugation techniques. Different types of density gradient media, like small hydrophilic organic molecules (e.g., sucrose), colloidal silica (e.g., Percoll™) or nonionic iodinated aromatic compounds (e.g., Metrizamide, Nycodenz®, OptiPrep™),[20] are used. A great variety of protocols have been published over the last 30 years. They differ in many details, e.g., gradient media, density gradient types or centrifugation forces.[21-24] However, the basic principles are the same in most protocols: during differential centrifugation, particles are separated, based upon their different size and density, by the stepwise increment of centrifugation forces. At the end of each centrifugation step, the resulting pellet and supernatant are separated and the pellet is applied to the next step of centrifugation. De Duve et al described a frequently cited method for the crude separation of organelles from liver tissue by differential centrifugation.[25]

To optimise the purity of lysosomal fractions, the product of the differential centrifugation is loaded onto a isopycnic density gradient. In contrast to rate zonal density gradient techniques based on the size, shape and density of the particles, the density and viscosity of the medium and the centrifugation forces, isopycnic centrifugation depends solely upon the buoyant density of the particle. What kind of medium or whether a continuous or discontinuous isopycnic density gradient is applied, depends on the experimenters' preference. Taken together, it has to be pointed out that lysosomes are of an extremely heterogeneous nature[26] and it is therefore not surprising that densities of lysosomes, mitochondria and peroxisomes overlap. Accordingly, changing the density or size of lysosomes might be a solution to the problem.

A promising and simple method for the preparation of lysosomes from livers of rats or mice is based upon the lowered density of lysosomes after a single intravenous injection of Triton WR 1339 (Tyloxapol).[27] This permits the separation of lysosomes from mitochondria and peroxisomes, representing the most frequent contaminations of purified lysosomal fractions. Triton WR 1339 is taken up by endocytosis and accumulates in lysosomes. By combining differential centrifugation steps[25] and an isopycnic discontinuous sucrose density gradient, tritosomal membrane preparations are almost freed of mitochondria, peroxisomes and microsomes. 10 – 40 % of total lysosomal enzyme activity is still found in these fractions which are 40 – 70-fold enriched in lysosomes.[28,29]

In addition, several other methods have been suggested in order to isolate lysosomes from tissue or cell culture by altering their density or size, depending on their content after specific loading, e.g., lysosomes filled with dextran (dextranosomes),[30] iron-loaded lysosomes[31] or colloidal gold-loaded lysosomes.[32] Glycyl-L-phenylalanine 2-naphthylamide (GPN) and methionine O-methyl esters (MOM), both representing substrates for lysosomal hydrolases, were shown to diffuse into lysosomes where specific degradation and accumulation of the products causes an osmotic rupture of the organelle. Due to their altered density after resealing, the lysosomes show an altered behaviour in an isopycnic density gradient and can be separated from organelles which normally cofractionate with the lysosomes.[33,34]

Quantitation of the Purity of Lysosomal/ Tritosomal Preparations

Regardless of which method was used to isolate lysosomes, the enrichment of lysosomes has to be determined and the rate of contamination by mitochondria or other organelles. The fastest and most convenient way to measure the enrichment of the lysosomes and to quantify contaminating organelles in a preparation are classical marker enzyme activity assays. For example, the enzyme succinate dehydrogenase is solely present in mitochondria. The presence of this protein in a lysosomal preparation indicates its contamination by mitochondria. For lysosomes, β-hexosaminidase, β-glucuronidase or acid phosphatase are frequently used marker enzymes. It is useful to determine the specific activities of different marker enzymes representing the entire set of organelles at every step of a preparation. By summing up all values, the balance of the preparation and the enrichment of the appropriate organelles in the final, lysosome-enriched fraction can be estimated. Furthermore, Western blots and, most notably, electron microscopy are useful techniques for estimating the purification efficiencies.

Solubilization of Lysosomal Membrane Proteins

Once the purity of the lysosomal membrane preparation is proven, membrane proteins have to be extracted from the lipid moiety and subsequently kept in solution. Detergents commonly used to extract and solubilise membrane proteins are: the strong anionic sodium dodecyl sulfate, the uncharged Triton X-100, and n-dodecyl-β-D-glucopyranoside, or zwitterionic CHAPS. These detergents are all amphipathic molecules containing both polar and hydrophobic groups mimicking the lipid-bilayer environment. After solubilisation, complete removal of the detergent could result in aggregation and may cause precipitation of membrane proteins, due to the clustering of hydrophobic regions and should be avoided.

1D/2D Gel Electrophoresis

The most widely used method in proteomics for analysing complex protein samples includes the separation of proteins by 2D gel electrophoresis (2D-GE), followed by the identification of single protein spots by mass spectrometry. In 2D-GE (Fig. 1), independently developed by Klose and O'Farrell[35,36] in 1975, proteins are separated in two sequential steps: in the first dimension on the basis of their net charge (isoelectric focussing), using ampholyte gradients or immobilised pH gradients (IPG) ,and in the second dimension based on their molecular weight through a polyacrylamide gel matrix (SDS-PAGE). After staining the gel, single protein spots are cut out from the gel and digested to short peptides by proteolysis with trypsin,

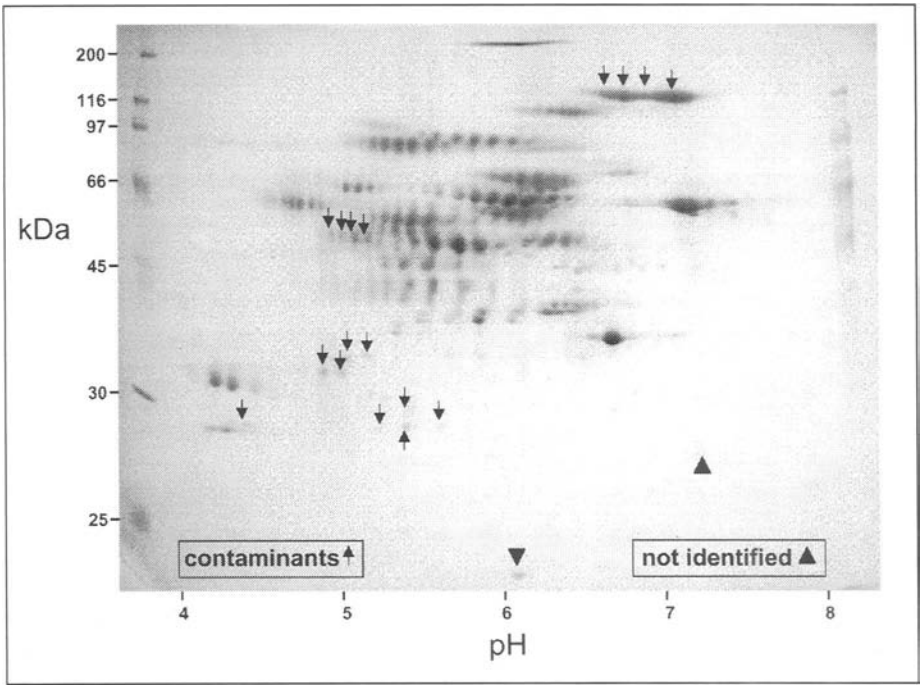

Figure 1. A separation of lysosomal matrix proteins by 2D gel electrophoresis. Lysosomal matrix proteins were isolated by mannose-6-phosphate receptor affinity chromatography. Mannose-6-phosphate tagged proteins were separated by 2D-GE by their charge in the first dimension and by their size in the second dimension. Subsequent analysis by mass spectrometry of the excised protein spots revealed approx. 25 lysosomal proteins in partially various isoforms reflecting the heterogenous post-translational modifications of the lysosomal proteins. Some protein spots were identified as contaminant proteins (marked with arrows) or could not be identified by mass spectrometry (marked with triangles).

which cleaves specifically at the amino acids lysine and arginine. Afterwards, the resulting peptides are identified by mass spectrometry.

The coupling of both separation techniques in 2D-GE, recent improvements in narrower focussing gradients and new staining dyes (e.g., Sypro-Ruby) led to a resolution power of up to 10000 proteins in a single gel.[37] However, 2D-GE comprises some limitations worth mentioning. High-throughput analysis of samples is difficult since the individual extraction, trypsin digestion and mass spectrometry analysis of each spot is time-consuming. Moreover, low abundant proteins and proteins with extreme pIs (<3.5 or >9.5) and masses (<15 or >150 kDa) normally escape detection.[3] Another critical point is the low dynamic range of 2D-GE, where low abundant proteins are hardly depicted next to high abundant proteins. Furthermore, solubilisation of some subsets of proteins in 2D-lysis buffer (7 M urea, 2 M thiourea and 4% CHAPS) might be inefficient, and less soluble proteins like hydrophobic integral membrane proteins are largely lost due to precipitation during isoelectric focussing (IEF), resulting in an inefficient transfer to the SDS gel. At a rough estimate, 30% of proteins are thought to be membrane proteins. By using 2D-GE, proteome studies of *Saccharomyces cerevisiae* identified only about 1% of integral membrane proteins.[38] This worrying problem can be overcome by eliminating the IEF step for proteomics of membrane proteins. The resulting simplification of the classical proteomics approach is based on simple SDS-PAGE which, in turn, complicates the analysis because of the low resolving power of 1D gel electrophoresis. After 1D-GE, the whole lane is cut into small sections and the proteins imbedded in each section are then in-gel digested into peptides which are then analysed by mass spectrometry (see below).[39]

In summary, 2D-GE is a powerful tool for elucidating the proteome of soluble lysosomal matrix proteins but has its limitations in the depiction of certain subsets of proteins.

Multi-Dimensional Protein Identification Technology

Recently, the multi-dimensional protein identification technology (MudPIT) was introduced by Yates and colleagues[40,41] in order to overcome the difficulties of gel-based methods and, in particular, the problems accompanying membrane proteins in 2D-GE. This promising gel-free approach takes advantage of the powerful separation properties of high-performance liquid chromatography (HPLC). Like 2D-GE, MudPIT represents a two-dimensional separation system but, here, the complexity problem is shifted from the protein level to the post-digestion peptide level. Briefly, membrane protein fractions are solubilised sequentially and the resulting protein mixtures are digested chemically and/or proteolytically.[41] The resultant complex mixtures of peptides are loaded on a biphasic HPLC system. First of all, the peptides are separated by their charge on a strong cation exchange (scx) column into subsets of peptides. A stepwise salt gradient elutes the subsets directly (in-line) into the second dimension where each subset of peptides is further separated according hydrophobicity on a reversed-phase (RP) column. As peptides elute from the second column, they are analysed directly by nano-electrospray ionisation tandem mass spectrometry (ESI-MS/MS). Peptide MS/MS spectra have to be judged properly and poor-quality spectra have to be deleted before the search for and identification of the respective proteins is performed within the appropriate protein database. Because of the high number of peptides analysed, only proper validation of the MS/MS spectra ensures the minimisation of false positive proteins. In addition, any protein should be identified by at least 2 – 5 different peptides, depending on the length of the peptides and the length of the protein, resulting in a peptide over protein ratio (protein coverage). Washburn et al[41] reported a large-scale analysis of the yeast proteome by MudPIT, where they identified 1484 proteins. They were able to detect and identify 131 membrane proteins with three or more predicted transmembrane domains, pointing out the unbiased character of MudPIT to some categories of proteins like extremes in abundance, pI, mass or hydrophobicity, which are critical in 2D-GE. MudPIT is an alternative to the traditional proteomic approach by 2D-GE followed by mass spectrometry, as it allows a higher sample throughput by automation and a resolving power comparable to 2D-GE. Moreover, there is a high flexibility in

different chromatography types coupled with each other (anion/cation exchanger, size exclusion, reverse phase, etc.). Although the MudPIT technique seems to overcome some of the limitations of 2D-GE, it also faces some notable problems: technical requirements are immense and, consequently, very costly. As mentioned above, proper validation of mass spectra is the most critical step to ensure the avoidance of false positive protein identification and is therefore very time-consuming.

Mass Spectrometry (MS)

Mass spectrometry is the key technology in proteome analysis since it enables the fast and sensitive identification of the separated proteins from 1D-/2D-GE, or peptides from MudPIT. This paragraph will briefly discuss the two most important mass spectrometry techniques for proteomics, namely matrix-assisted laser desorption-ionisation (MALDI) and electrospray ionisation (ESI).[42]

Mass spectrometry of macromolecules larger than 1000 Da was developed by two independent groups in 1987. Although Koichi Tanaka's method[43,44] was awarded the Nobel prize for chemistry in 2002, it is not used practically. Some months later Hillenkamp and Karas established a technique called matrix-assisted laser desorption ionisation mass spectrometry (MALDI-MS)[45,46] which is in use in many laboratories worldwide. In MALDI-MS a protein or peptide sample (analyte) is embedded in an UV-absorbing matrix consisting of small organic molecules. After cocrystallisation of matrix and analyte, under vaccum conditions a pulsed laser beam is applied for nanoseconds to the cocrystallized sample. Due to the applied energy, matrix and analyte molecules (peptides or proteins) are released into the gas phase and are accelerated by an electrical field into the mass analyser. In MALDI this is usually a time-of-flight analyser (TOF) where the released and accelerated samples pass through a field-free region, where they are separated by differences in their mass/charge (m/z) ratio. At the end of the field-free region, a detector measures the incoming ionised analytes, where heavy ions with a high mass/charge ratio arrive later than light ions. Former calibration of the MALDI-TOF with reference molecules, and the fact that the mass and charge of a molecule are proportional to the square of the time-of-flight, allows the determination of the mass of any molecule. In a traditional proteomic approach, single protein spots are excised from a 2D gel, digested to peptides and analysed by MALDI-TOF, resulting in mass profiles (or fingerprints) of a peptide pool.[47] These experimental mass profiles are then compared with the theoretical peptide profiles of a protein data-base. However, MALDI-TOF has some limitations, since mass fingerprint needs sufficient database information about protein variants and is sensitive to mixtures of more than two or three proteins.

When coupled with HPLC, electrospray ionisation mass spectrometry (ESI-MS) allows the identification of proteins, even from a complex mixture of proteins or peptides.[48] In ESI, the proteins or peptides are also ionised but in a different manner than in MALDI. A solution of proteins or peptides—sometimes coming from a low-flow-rate HPLC like in MudPIT—enters the ESI-MS through a fine capillary. Applying a strong electric field between capillary tip and mass spectrometer inlet results in a spray of droplets containing few or even only one ionised macromolecule. ESI is generally coupled with quadrupole mass analysers and ion traps (for further information see ref. 42).

Tandem mass spectrometry, another type of MS, even allows peptide sequencing. In this approach, a selected ion from a mixture in the first mass spectrometry analysis is fragmented within a collision cell and the resulting fragments are passed into a second mass analyser, from where the peptide sequence can be deduced.[42]

Bioinformatics for Proteome Analysis

Proteomic techniques like MudPIT or 2D-GE, coupled with mass spectrometry, provide huge quantities of data in a single experiment. Automatic interpretation of mass spectrometry data, e.g., mass fingerprints and fragmentation spectra, have become essential for

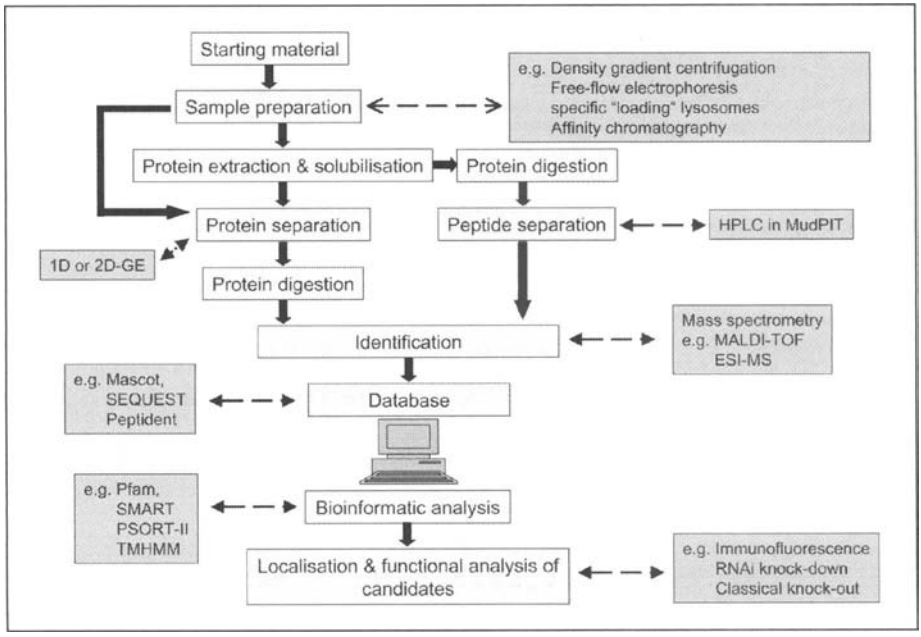

Figure 2. A schematic diagram outlining the different strategies for lysosomal proteomic approaches. To analyse the lysosomal membrane proteins, starting material might be tissues rich in lysosomes, e.g., liver or placenta. Hence lysosomes have to be isolated from other organelles by different separation techniques (see: Sample preparation for lysosomal membrane proteins). The analysis of lysosomal matrix proteins starts with the supernatant of cells secreting mannose-6-phosphate tagged proteins. The latter are then purified by mannose-6-phosphate receptor affinity chromatography. Prior to the analysis of the proteins/peptides by mass spectrometry, the proteins are either separated by gel electrophoresis techniques and are subsequently digested. Alternatively proteins are directly digested into peptides which are separated by high-performance liquid chromatography (HPLC). Peptide mixtures, which have been separated by a variety ways, are analysed by mass spectrometry.

high-throughput processes aiming to study proteomes (Fig. 2). In the early nineties, the first computer algorithms using mass maps to search for and identify proteins in protein databases were developed. Each protein in the database is in silico digested by site-specific enzymes, like trypsin, and predicted mass/charge (m/z) resultants are then compared to experimental m/z values. If peptide sequence analysis by tandem mass spectrometry is used, this sequence information can consequently be exploited to search protein or even nucleotide databases such as the expressed sequence tag (EST) databases, in particular those of well sequenced organisms. Frequently used algorithms for protein identification in proteomics by peptide masses include PepIdent, PepFrag, Prospector, Mascot and SEQUEST (reviewed in refs. 49, 50). The most commonly used protein-sequence databases are UniProt and the nonredundant collection of protein sequences at the National Center for Biotechnology Information (NCBI). After the identification of a possibly novel protein from MS data, a protein function prediction is performed. Sequence alignments by BLAST (NCBI) can yield information about homologues and orthologues. Functional domains can be predicted by tools like Pfam, SMART, Interpro or PROSITE (reviewed in refs. 49, 50). For subcellular proteomics, the intracellular localisation of proteins plays a major role and can be foretold by PSORT-II[51] to a certain extent. SignalP[52] can predict the presence and location of signal peptides and cleavage sites. TMHMM[53] can identify transmembrane helices in proteins very reliably.

Validation of Proteomics Results and Perspectives

Bioinformatics can help to annotate the results of proteomic experiments. Nevertheless, these results have to be confirmed by traditional cell biological or biochemical techniques. The consequence of a lysosomal proteome approach is that newly identified proteins have to be shown to be truly associated to the lysosomal compartment. This can be done by creating tagged cDNA versions (His-, Flag-, Myc-Tag, etc.) or fusion constructs with green-fluorescent protein and subsequent expression in appropriate cell systems like HeLa or COS. However, the high expression levels of tagged proteins which occur sometimes may lead to misfolding and, consequently, incorrect localisation of the investigated protein. Hence, raising antibodies against the protein is preferable, as they allow the investigation of subcellular localisation on endogenous protein levels. Alternatively, due to their obligate mannose-6-phosphate residue, newly identified lysosomal matrix proteins should bind to a mannose-6-phosphate receptor in affinity chromatography approaches, thus confirming their lysosomal sorting signal. For that purpose, tagged versions of the candidates can be transfected into eukaryotic cells. After the stimulated secretion of lysosomal matrix proteins, by ammonium chloride and M-6-P receptor binding, the tagged candidate should elute with the lysosomal matrix protein fraction. For functional analysis of the candidate proteins, RNA interference (RNAi) can be performed and classical knock-out mice should be generated. RNAi has the danger of failing in functional analysis, in particular, in lysosomal matrix proteins, since this approach is rather a knock-down than a knock-out approach but, for many lysosomal hydrolases, residual enzyme activities of 2% are sufficient to prevent lysosomal storage.

Another future aspect of proteomics will be the differential analysis of a pathological proteomes versus the proteomes of healthy individuals. Such approaches are based on the comparison of 2D-GE images of both proteomes. Differences in the expression pattern or expression level depicted in 2D-GE might give insights into the dependency, regulation and compensation of lysosomal proteins in health and disease.[54,55]

Transcriptome Analysis of Lysosome Functions

Proteome oriented techniques can be used to directly assess which proteins are localised in organelles using biochemical organelle purification and subsequent protein detection techniques (cf. first section of this chapter). Transcriptome analysis is a rather indirect approach to the question of organelle function because the measured entities are mRNA molecules. Their cellular localisation is of interest for several biological processes, e.g., early development in several organisms,[56] the differentiation of cytosolic and membrane associated transcripts[57] or nuclear mRNA export. But in the context of lysosomal function, mRNA localisation is not of interest and a different strategy is required. Gene expression profiles reflect the overall transcriptional reaction of the cell to the conditions it is subjected to. They can be used to study differential gene expression following an experimental stimulus or to describe basal gene expression in a biological process, e.g., development or ageing. The analysis of organelle function using gene expression profiling can be performed by perturbing its function and subsequent measuring of the effect of the perturbation. This approach has been described for peroxisome assembly in yeast.[58] Smith et al induced peroxisome proliferation and measured the transcription response to this event. Similar approaches can be used to study lysosomal function.[59] The following sections briefly describe the principle of transcriptome analysis and strategies to analyse lysosomal function.

Principle and Application of Transcriptome Analysis

Experimental Procedures

Techniques to analyse gene expression on the mRNA level have evolved since the 1970s. Among them, hybridisation techniques using the base pairing property of complementary nucleic acid molecules evolved rapidly since the development of the Northern Blot[60] in which

a soluble, radioactively labelled cDNA probe is hybridised to a size separated, membrane-bound (immobilised) mRNA-target to detect the size and abundance of one transcript binding the probe. First steps to reverse the principle of the Northern Blot were undertaken soon: instead of immobilising the mRNA-targets on a membrane and labelling one cDNA-probe that is hybridised in solution (Northern Blot), multiple cDNA-probes were immobilised as spots on a membrane (macroarrays, early 1990s) or on glass (microarrays, late 1990s). Companies developed microarrays with oligo-nucleotide-probes of differing qualities and lengths. Affymetrix produces oligo-arrays with short oligos using in-situ photolithography, while Agilent manifactures long-oligo-arrays with an ink-jet-nucleotide linking technique. Instead of using a blotted mixture of targets, a mixture of multiple mRNA-targets were labelled with radioactivity (membrane arrays, ref. 61) or fluorescence (glass arrays, ref. 62) and hybridized in solution. Using labelled targets in solution and immobilised probes spotted as arrays, the expression of thousands of genes can be monitored at a time by measuring the radioactivity/fluorescence signal at every spot. The mRNA targets can be labelled using reverse transcriptase incorporating labelled nucleotidetriphosphates as substrate[63,64] or with the help of more sophisticated methods (e.g., amino-allyl-dye-coupling, FairPlay, Stratagene). If radioactive labelling or single-colour-oligonucelotide-microarrays (Affymetrix) are used, only one colour is available and direct comparisons of two different mRNA-targets on a single array can not be performed. With fluorescent labelling, two different fluorescent dyes can be used for different mRNA-targets enabling direct comparisons of the targets on one microarray. Because the experimental variance between different arrays is quite high due to varying experimental factors, e.g., labelling efficiency and hybridisation quality, direct comparison approaches using the statistical block principle are to be preferred.[65] In experiments designed according to the block principle, the setting is optimised to measure only the effect of interest while excluding other sources of variance. For microarrays, this means that samples of two different treatments are compared on a single array to avoid confounding of the treatment effect with the experimental variance associated with the hybridisation of the array.

It is of high interest to obtain microarrays covering as many probes (genes) as possible because in a given experimental situation, the investigator cannot know in advance which genes will be differentially expressed after a stimulus. Therefore, since the development of microarrays, one major goal has been to obtain arrays that cover the entire transcriptome of the organism that is to be studied, i.e., all its transcriptional entitites (expressed genes). So far, this has only been achieved for a few organisms for which all the expressed genes are known; to this end, the complete genome sequence clone is insufficient. But with the rapid advances in genome and EST-sequencing, this will be possible for more species soon.

Eisen and Brown[63] give a detailed description of the experimental process of using microarrays. In summary, to perform a labelling for microarrays, the targets (total RNA or mRNA) must be prepared from tissues or cells. Total RNA is usually treated with DNAse to remove genomic DNA that can inhibit the labelling reaction and lead to increased image background. Total RNA or mRNA is then subjected to reverse transcription in the presence of a fluorescent labelled deoxycytidine (or -uridine) triphosphate and a low-C (or -T)-dNTP mixture. The resulting cDNA contains nucleotides with a fluorescent label. On glass microarrays using fluorescent dyes, two separate labelling reactions with distinct fluorescent dyes are used per array. After removal of nonincorporated dyes, the two reactions are combined, hybridisation buffer is added and a cohybridization of both targets to the microarray is performed over night. After washing the arrays, image data are acquired by scanning the arrays using laser light to induce the two fluorescent dyes at two different wavelengths. Figure 3 shows a typical microarray rainbow-color-image of one channel. The fluorescent signal measured at every spotted probe is roughly proportional to the amount of mRNA present in the target. This means that microarrays are semi-quantitative, i.e., the direction of gene expression differences measured on microarrays are correct, but their magnitude may be over- or underestimated.

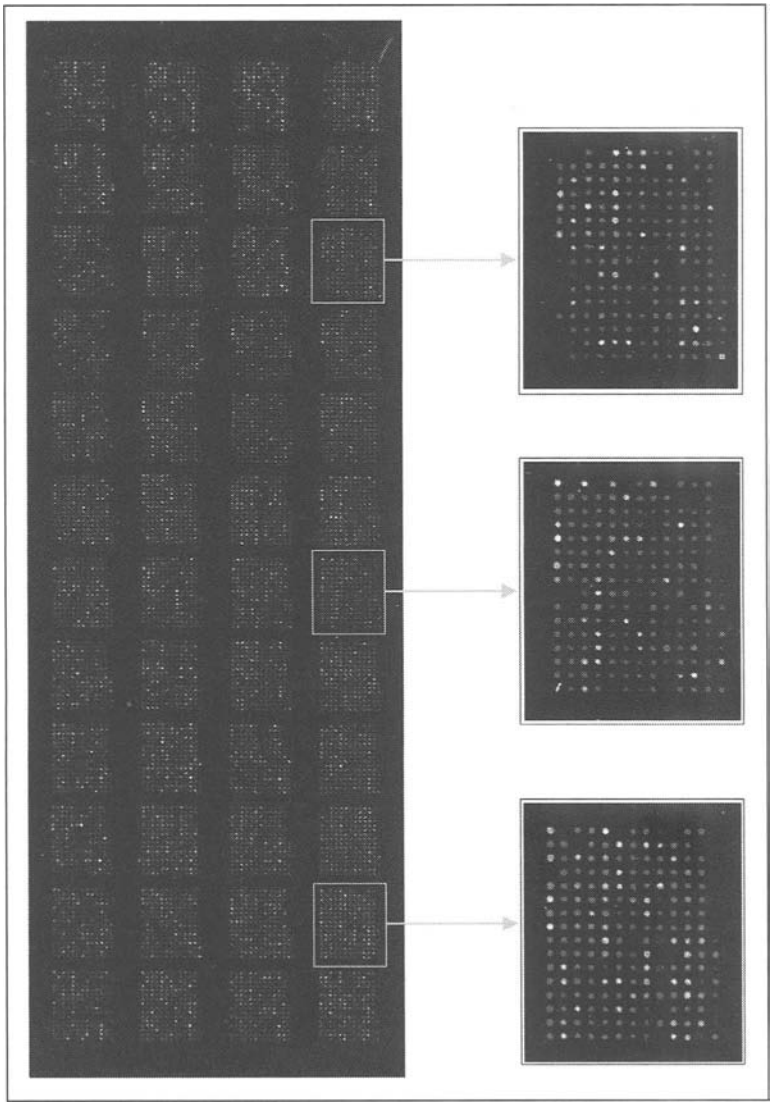

Figure 3. Picture of a typical cDNA-microarray with approx. 10.000 spots. The RNA target was labelled and hybridised to the array as described in Eisen and Brown.[63] One channel (532 nm) is shown in rainbow-falsecolors (blue—low signal, red/white—highest signal). The magnifications on the right side demonstrate the hybridization dynamics reflected in different signal intensities. A color version of this figure is available online at http://www.Eureka.com.

Data Analysis and Annotation

Image data are transformed to numerical data using pattern recognition methods implemented in image analysis software.[66] The numerical data have to be processed and analysed using statistical methods. This complex and demanding task has led to a research field of its own (overview: ref. 67). The main tasks are data normalisation,[68,69] testing for differentially

expressed genes,[70] gene expression pattern recognition[71] and classification.[72] Data normalisation is required to eliminate hybridisation artefacts and differing brightness and incorporation of the two fluorescent dyes used for labelling. It allows the computation of statistics involving several microarrays.[69] Testing for differentially expressed genes is often the primary goal of microarray experiments. It is done to obtain genes that vary between two or more experimental conditions. Various approaches have been proposed to this end and are still under improvement.[70,73] If pattern recognition or classification methods are used, genes forming a pattern or classifying genes are obtained.

In all three situations, the output of the statistical methods consists of gene lists. These lists are often difficult to interpret as it is not easy to deduce the biological meaning of the gene regulation pattern that is observed. Bioinformatics have to be used to annotate these lists with gene names and functions[74] using gene classification and function description repositories such as Gene Ontology[75,76] or Uniprot[77] as well as links to the literature.[78] Furthermore, the interpretability of such lists can be improved using pathway annotation, i.e., the embedding of gene expression data in known cellular biochemical pathways and metabolic networks.[79,80] In single-cell organisms as *E. coli* or *S. cerevisiae*, the data can be used to improve the description of transcriptional regulation networks.[81]

To validate the microarray data and to draw conclusions, the differentially expressed genes that contribute most to the biological interpretation of the data have to be confirmed using an independent method, e.g., Real-Time-PCR or Northern Blot.

As gene expression is tightly controlled at every step from transcription to posttranslational modification, only 60% of the differences in mRNA levels are found at the protein level. This has to be considered when evaluating microarray data and the ultimate step of every analysis should be the validation of results on the functional protein level.

Gene Expression Profiling to Analyse Lysosomal Functions

The analysis of lysosomal function using gene expression profiling can be performed by function perturbation and subsequent measuring of its effects. This approach has recently been applied by the group of Mark Sands.[59] The study used a mouse model for Mucopolysaccharidosis VII (MPS VII), a lysosomal storage disease in which the lysosomal function is perturbated by the deficiency of a catabolic hydrolase, β-glucuronidase, which is essential for the degradation of glucosaminoglycans (cf. Chapter 6). The authors tried to reverse the functional perturbation using enzyme replacement therapy. They compared the expression profiles of treated mutants to those of untreated mutant and wild-type animals in liver at two different times points. The treatment effect was estimated and the authors demonstrated that the treatment scheme used in the study could eliminate lysosomal storage in the mutants, but it could not fully reverse the alterations in gene expression caused by the disease. This indicates that the therapy-resistent transcriptional changes could be related to secondary alterations. The deepened analysis of these changes can help to understand how the lysosomal storage influences the cells and leads to the secondary pathology that is not directly related to the storage. The authors also identified changes in biochemical pathways that were previously not known to be related to MPS VII. They pointed out that the analysis of transcriptional changes enables one to discern transcriptional from translational regulation of proteins known to be dysregulated in lysosomal storage diseases. Taken together, this study demonstrates how function perturbation studies can be used to give new insight into the function of lysosomes in the cell. The approach can be applied to the whole spectrum of lysosomal diseases using mouse models as well as human cells with and without perturbation reversal by enzyme treatment. It will be interesting to see to which degree different models of lysosomal storage diseases show common features and could allow conclusions with regard to general principles in lysosomal storage.

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CHAPTER 12

External Lysosomes:
The Osteoclast and Its Unique Capacities to Degrade Mineralised Tissues

Vincent Everts* and Wouter Beertsen

Introduction

Lysosomes contain the enzymatic machinery to digest a host of organic substances. This degrading activity occurs primarily inside the living cell where the lysosomes reside. Yet, some cells use lysosomal enzymes in the extracellular space to digest proteins in their direct vicinity. Secretion of these enzymes by neutrophils is an essential step in the digestion/killing of (parts of) bacteria in the defence of an organism.[1-4] Macrophages also may secrete lysosomal enzymes.[2,5-7] Some observations even suggest that fibroblasts have the capacity to secrete such enzymes into the extracellular space.[8,9] Since the majority of lysosomal enzymes are active at a low pH, an effective use of the enzymes extracellular can occur only if a low pH is created and maintained for some time. This is possible if the cell has the capacity to seclude a restricted area from its surrounding environment. The latter situation is highly effective during degradation of mineralised matrices, like bone and calcified cartilage. The cell primarily involved in this process is the multinucleated osteoclast.[10,11] In the present chapter some of the unique properties of this cell will be discussed, in particular its ability to create an extracellular lysosomal compartment, the prime site where resorption of mineralised tissue occurs.

The Osteoclast as a Highly Polarized Proton-Secreting Cell

General Description of the Osteoclast and Its Genesis

The osteoclast has several features making it rather unique. The cell is not only one of the few multinucleated cell types in our body, but it also exhibits certain characteristics that are normally reserved for proton-secreting epithelia. In 1992 Gluck[12] considered the "osteoclast as a unicellular proton-transporting epithelium". Characteristic for such epithelial cells are (i) a functional polarity, (ii) an extremely high number of mitochondria, (iii) a high carbonic anhydrase II activity, and (iv) the secretion of protons.[13] Each of these characteristics is also apparent in osteoclasts.

The osteoclast is formed by fusion of mononuclear precursor cells originating from the lineage of monocytes. Under the influence of the cytokine receptor of activator nuclear factor κB-ligand (RANKL), primarily produced by cells of the osteoblast lineage, a subset of RANK-expressing monocytes fuse and form in the vicinity of the bone surface a multikaryon, the osteoclast.[10,11] Following its formation it strongly attaches to the bone surface; an attachment mediated by matrix protein-binding membrane receptors, the integrins. Of this family of integrins, osteoclasts and their precursors express in particular the $\alpha_v\beta_3$ integrin,[14-16] an integrin that recognizes RGD-sequences of proteins such as osteopontin and bone sialoprotein.

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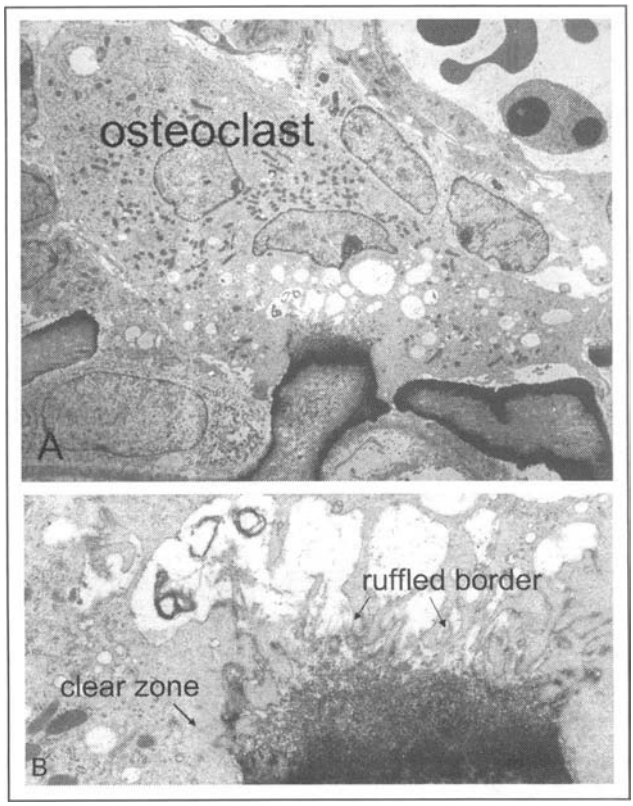

Figure 1. Electron micrograph of an actively resorbing osteoclast adjacent to calcified cartilage. The ruffled border area is shown at a higher magnification in B. Note the clear zone by which the osteoclast attaches to the calcified matrix and separates its surrounding environment from the "external lysosome", the ruffled border.

Relatively high levels of osteopontin are present in the bone matrix and, under certain conditions, also along the bone surface. This is particularly the case at sites were resorption is initiated.[17] Prior to attachment of a (pre)osteoclast to the bone surface osteoblast-like cells clean the surface from nonmineralised constituents like collagen fibrils.[18-20] This cleaning activity probably results in exposure of bone-associated proteins like osteopontin to which the osteoclast may subsequently attach. After initial attachment, the osteoclast isolates part of the bone surface from the surrounding environment. A circular sealing or clear zone seals off this area. The sealing zone is characterized by an absence of cytoplasmic organelles, but it contains a high density of microfilaments. At numerous sites the microfilaments concentrate and attach to the plasma membrane, forming a ring of attachment sites, the podosomes.[21] The receptors involved in the attachment of the membrane to the bone surface have still not been identified. Some authors claim the presence of $\alpha_v\beta_3$ integrins, whereas others were unable to detect this integrin in the sealing zone region (see ref. 22). Although the thus formed barrier is taken to prevent leakage of bone resorbing compounds (protons and proteinases) to the cell's surrounding, negatively charged molecules with a MW up to 10,000 have the capacity to diffuse into the sealed area.[23] In the central portion of this area, a unique region is formed where the actual resorption of the mineralized tissue is initiated and accomplished. This central area is the alleged ruffled border (Fig. 1) and its formation makes the osteoclast a highly polarized cell. In a

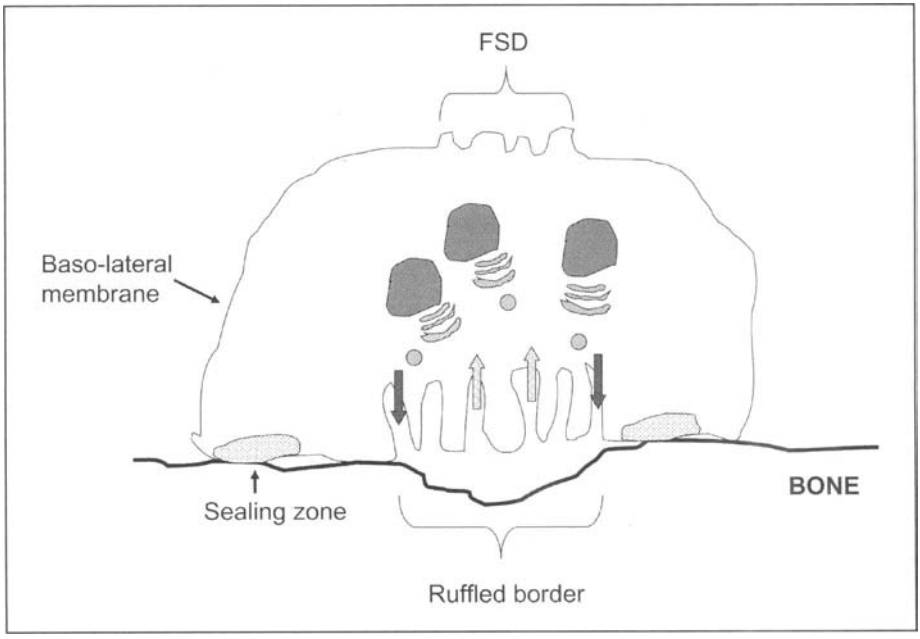

Figure 2. Schematic presentation of the polarity of an osteoclast. According to recent studies 5 different membrane domains can be recognized: (i) the sealing zone, (ii) the baso-lateral membrane, (iii) the functional secretory domain (FSD), and the ruffled border membrane with two domains, (iv) the lateral exocytotic part and (v) the central endocytotic domain (indicated by arrows).[25,26,91]

series of elegant studies Väänänen and colleagues showed the osteoclast to have at least five different membrane compartments: (i) the sealing zone, (ii) the baso-lateral membrane, (iii) the functional secretory domain (FSD), (iv) the lateral region of the ruffled border membrane, and (v) the central portion of the ruffled border[24-26] (Fig. 2).

The Ruffled Border Membrane Limits the External Lysosome

A strongly undulating membrane characterizes the ruffled border. At the electron microscopic level it differs from the other plasma membrane areas of the osteoclast, e.g., the sealing zone and baso-lateral membrane. It is somewhat thicker, suggesting a different composition, which proves indeed to be the case. Numerous data indicate profound similarities between the ruffled border membrane and that of lysosomes.[27-30] One of the first direct clues suggesting the lysosomal origin of the ruffled border membrane was the finding of the presence of a membrane-associated pump, H^+-V-ATPase.[27] This class of pumps is highly abundant in lysosomal membranes and is crucial for maintaining a low pH within these structures. In addition to V-ATPase, the membrane of the ruffled border contains a number of other lysosomal membrane proteins, among which LAMP-1 (LGP107), LAMP-2 (LGP96), and LIMP-2 (LGP85).[31,32]

A series of observations indicate that endosomal/lysosomal vacuoles are transported to the site of resorption and collectively form the external lysosomal membrane: the ruffled border.[25] Prior to the appearance of the ruffled border, the osteoclast contains high numbers of vacuoles of low pH which fuse with the newly forming membrane[25] upon attachment to the bone surface. Recently it was shown that the vacuoles fuse mainly with the lateral parts of the ruffled border membrane, whereas the central part appears essential for endocytosis of bone matrix remnants (see below).[26] The transportation of the lysosomal vacuoles depends on members of the ras super family (the Rabs), which are proteins that regulate intracellular and plasma membrane fusion

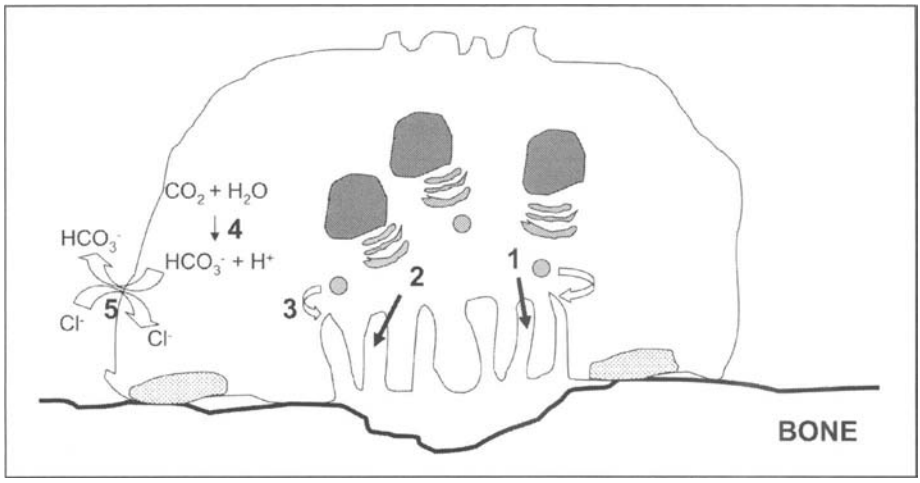

Figure 3. Schematic presentation of the processes involved in the acidification of the ruffled border area. 1) H^+-V-ATPase in the ruffled border membrane mediates transport of H^+. 2) Chloride channel transports chloride to the external lysosome for electro-neutrality. 3) Lysosomal transport vesicles deliver membrane constituents and enzymes to the ruffled border. 4) Carbonic anhydrase II mediates the generation of protons. 5) Chloride-bicarbonate exchanger (see ref. 92).

and expansion. An important member of this group involved in the transport within the osteoclast is Rab7[33] probably in conjunction with Rab3.[34] During these events the membrane-residing protein c-Src proved to be essential for keeping the osteoclast in a polarized state.[35]

Two Major Functions of the External Lysosome: Acidification and Proteolysis

Acidification (Fig. 3)

Resorption of mineralised tissues depends on two major activities: i.e., demineralisation and digestion of organic matrix constituents. Demineralisation of the hydroxylapatite crystals is effectuated by the low pH in the resorption area. Some authors noted a pH in this area of around 4-5, comparable to the pH found in lysosomes.[27,36] The V-ATPase located in the ruffled border membrane modulates this acidification process as was shown by (i) blocking the activity of V-ATPase with compounds like bafilomycin,[37] (ii) interfering with its expression,[38] and (iii) by deletion of its gene.[39] All these studies have unequivocally demonstrated that without a proper functioning of V-ATPase, bone degradation is blocked.[37-40] In humans it was shown that absence of these pumps resulted in a severe form of osteopetrosis.[39,40] In this condition osteoclastogenesis appears unaffected, but the cells lack the capacity to resorb bone. Although they still can attach to the bone surface, a ruffled border is not formed, thus suggesting that expression of the pumps is crucial for the formation of the ruffled border.

Chloride channels, present in the same membrane areas that harbour V-ATPase, provide for electro-neutrality. Recently is has been shown that the chloride channel Cl7C is essential to this process of electro-neutrality.[41] When this membrane pump lacks, a severe osteopetrotic condition will develop. This was demonstrated both in mice lacking the chloride channel, and for one of the forms of osteopetrosis occurring in humans.[41]

The H^+ needed for acidification is provided by the activity of carbonic anhydrase II. This enzyme catalyses the hydration of CO_2 to bicarbonate and a proton, thus contributing to H^+ secretion for acidification and to HCO_3^- secretion by the HCO_3^-/Cl^- exchanger (Fig. 3).[42] Carbonic anhydrase II is abundant in the cytoplasm of the osteoclast and on the inner surface

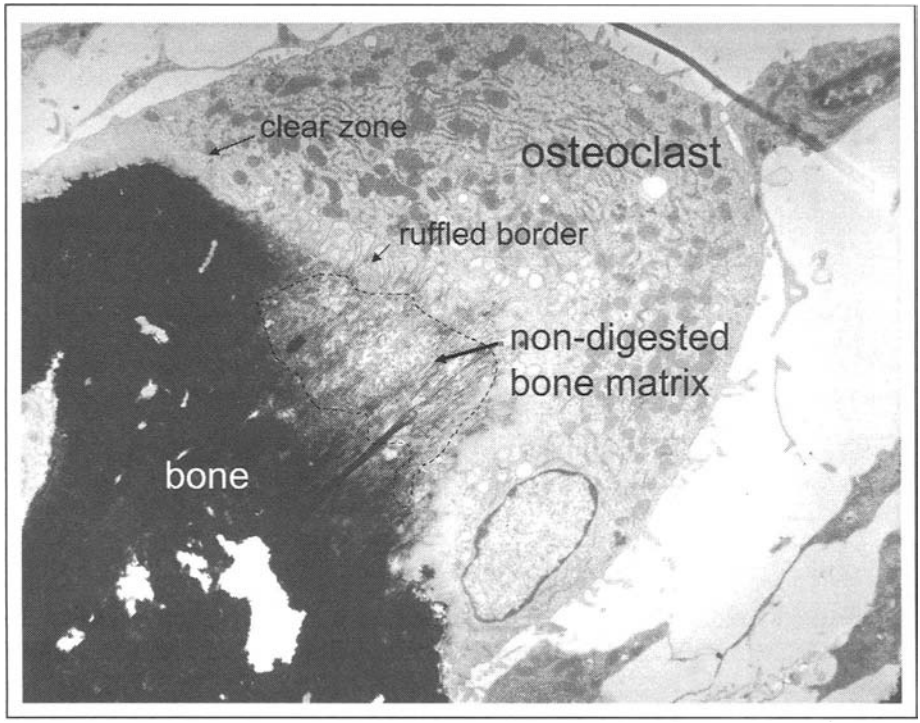

Figure 4. Effect of a selective inhibitor of cysteine proteinases (E-64), on osteoclast-mediated digestion of bone matrix. Note the large (outlined) area of nondigested demineralised bone matrix adjacent to the ruffled border of the osteoclast. This phenomenon is characteristic for bone in which the osteoclasts lack cathepsin K activity (e.g., pycnodysostosis[57] and cathepsin K deficient mice).[60,61]

of the ruffled border. Lack of the enzyme results in the inability of osteoclasts to resorb bone and leads to osteopetrosis (reviewed in ref. 43).

It is of interest to note that in most cases where the osteoclast is not able to lower the pH, e.g., due to lack of membrane pumps and/or carbonic anhydrase II, the formation of a ruffled border is prevented, whereas absence of proteolytic enzymes crucial for the second step, the resorption of matrix constituents (see below), does not block ruffled border formation. Under these conditions the osteoclasts have a relatively normal appearance. They attach to the bone surface and a ruffled border is clearly formed. These observations highlight the essentiality of the first step in mineralised tissue resorption, the ability of osteoclasts to create an acidified extracellular milieu.

Proteolysis

Cysteine Proteinases (Figs. 4, 5)

Following demineralisation, the next major step in the resorption process is the digestion of the bone matrix, which is mainly collagenous in nature but also contains numerous minor components like proteoglycans and glycoproteins. The collagens are known to be extremely resistant to digestion. Only a few enzymes have the capacity to degrade this protein. Among these are some members of the family of matrix metalloproteinases and a few belonging to the group of cysteine proteinases.[11] The latter group of enzymes is of particular interest since most of its members are present in lysosomal vacuoles and exert their activity at a low pH. Moreover, these enzymes have a mannose 6-phosphate recognition site and ample data indicate

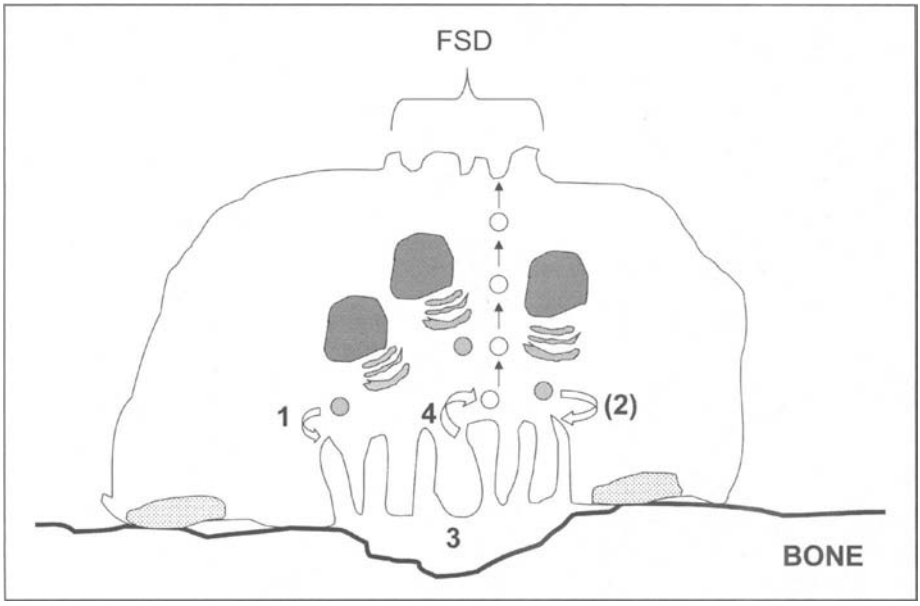

Figure 5. Schematic presentation of the degradation of bone matrix by the osteoclast. 1) Lysosomal vacuole containing proteolytic enzymes, in particular the cysteine proteinase cathepsin K, releases its content in the ruffled border area. 2) In certain subsets of osteoclasts matrix metalloproteinases are released and involved in matrix digestion. 3) Actual degradation takes place in the ruffled border area. The bulk of the bone matrix is digested by cysteine proteinases (and matrix metalloproteinases). 4) Endocytosis of partially digested bone matrix proteins. These proteins are further digested in the vacuoles. One of the enzymes assumed to play a role in this process is tartrate resistant acid phosphatase (TRAP). Remnants are subsequently released at the functional secretory domain (FSD).

transportation of lysosomal enzymes by the cation-independent mannose 6-phosphate receptor to the ruffled border membrane of actively resorbing osteoclasts.[44,45]

The first direct indications that cysteine proteinases are indeed essential for osteoclastic bone resorption were provided by Delaissé and coworkers.[46,47] They showed that selective inhibition of the activity of cysteine proteinases by using enzyme inhibitors strongly decreased bone resorption. In subsequent studies it was shown that in the presence of such inhibitors the osteoclasts had the capacity to demineralise bone but not to digest its organic matrix (Fig. 4).[48,49] Yet, the data did not provide insight which enzyme (or enzymes) was (were) actually responsible for the digestion.

The most potent collagenolytic cysteine proteinases are the cathepsins B, L and K. Immunolocalization and enzyme histochemical studies have indicated the presence and activity of one or more of these enzymes in osteoclasts and/or the ruffled border area.[50-54] Although the role of the different cysteine proteinases in the digestion of bone matrix is still not entirely clear, there is convincing evidence for at least one of them: cathepsin K, formerly called cathepsin O2.[55,56] A relatively rare osteopetrosis-like disease, pycnodysostosis, provided one of the first (indirect) clues for the role played by this enzyme. Analysis of bone biopsies from patients suffering from this disease revealed the presence of large areas of demineralised yet nondigested bone matrix adjacent to the ruffled border of osteoclasts.[57] This observation suggested that the osteoclasts had the capacity to demineralise bone but lacked the ability to degrade its matrix. In a subsequent study it was shown that selective inhibition of cysteine proteinase activity resulted in a similar phenomenon; again large areas of nondigested demineralised bone matrix were found facing the ruffled border of the osteoclasts (Fig. 4).[48] Later, genetic analyses by Gelb and

coworkers[58] and Johnson et al[59] showed in pycnodysostosis patients mutations in the cathepsin K gene. Together these data strongly suggested that a lack of cathepsin K was the cause of a decreased osteoclastic bone resorption in pycnodysostosis. Subsequent studies have indeed provided conclusive evidence for this assumption. First, Saftig and coworkers[60] and Gowen et al[61] generated mice that lacked cathepsin K. These mice showed an osteoclast phenotype similar to the one described for pycnodysostosis; large areas of nondigested demineralised bone matrix were apparent adjacent to the ruffled border of the osteoclasts. The inability of the osteoclasts to properly digest the bone matrix provides an explanation for the osteopetrotic features seen in man and mice. Yet, in both species the osteopetrotic features are relatively mild, suggesting that the inability to properly degrade bone matrix can be overcome to a certain extent. Recently, we have been able to provide an explanation for this. The areas of bone matrix not digested by the osteoclasts appeared to be populated by bone lining cells. These cells were able to engulf and subsequently digest the bone matrix remnants left by the osteoclasts.[20] A second series of arguments for a role of cathepsin K in bone matrix resorption was provided by the use of selective blockers of this enzyme or by interfering with its expression.[62-64] Again bone resorption was significantly reduced. Collectively, the essential part played by cathepsin K in osteoclastic bone matrix resorption now seems obvious.

In addition to cathepsin K other cysteine proteinases have been suggested to be involved in bone matrix digestion as well.[65-67] Among the most likely candidates are cathepsin B and L. Yet, conclusive evidence for their participation is still not available. Using selective inhibitors of these enzymes, a decreased resorption of bone was found in various in vitro models.[48,68] It is also noteworthy that these enzymes have been immunolocalized in the osteoclast.[51,53] Bone resorption, however, seems unaffected in mice deficient for either of the two cathepsins (Everts V, Saftig P, Korper W, and Beertsen W, unpublished). Yet, the morphology of osteoclasts in mice lacking cathepsin B differs from osteoclasts in wild type animals in that relatively high numbers of cytoplasmic vacuoles are present. This may point to an intracellular role played by the enzyme. Hill et al[68] proposed participation of cathepsin B in intracellular digestion of proteins internalized by the osteoclast. Cathepsin L, on the other hand, was assumed to be active in the extracellular -ruffled border- area.

In line with a possible contribution of cathepsin B in intracellular degradation is the finding of transcytosis by osteoclasts.[69,70] Following partial digestion of bone matrix protein, osteoclasts ingest a fraction of this material which is subsequently (partly) exocytosed at the opposite side of the cell, at the functional secretory domain (Fig. 5).[25] Although direct proof for a role of cysteine proteinases in this intracellular process is still lacking, support is given by the following observations. In the absence of the activity of cathepsin K high amounts of nondigested bone collagen fibrils are found in lysosomal vacuoles of the osteoclast.[57,71] It is not known, however, whether this accumulation represents material normally digested intracellularly or an accumulation of proteins that can no longer be digested due to improper degradation in the extracellular space. Previously it was shown that cysteine proteinases play an important part in the digestion of noncollagenous bone matrix components (e.g., proteoglycans).[72] These molecules may interfere with an effective digestion of collagen extracellularly as a result of which they are endocytosed and accumulate inside the cell.

Taken together involvement of cysteine proteinases in osteoclastic resorption of bone matrix has convincingly been demonstrated. Whether beside cathepsin K other members of this class of proteinases are involved, needs to be clarified.

Acid Phosphatases

Apart from cysteine proteinases, osteoclasts express high levels of another group of lysosome-associated enzymes, the acid phosphatases. One member of this group is tartrate resistant acid phosphatase (TRAP or TRACP). TRAP is highly expressed particularly in osteoclasts and therefore generally used as a marker enzyme for this type of cell. Two isoforms have been described for TRAP, the 5a and 5b form. The isoform expressed by osteoclasts (which is

thought to be quite unique for this cell) is TRAP 5b.[73] The 5a isoform is expressed by other cells, among which the osteoblast.[74]

In spite of the long known fact that osteoclasts are rich in TRAP, it is only recently that functions of this enzyme have been discovered. First, it was found that the enzyme is able to dephosphorylate bone-associated proteins like osteopontin.[75,76] Subsequent studies have demonstrated that mice deficient for TRAP exhibit a relatively mild osteopetrotic phenotype,[77] thus indicating that in the absence of the enzyme, bone remodelling is hampered but not totally blocked. Osteoclasts in these mice showed a somewhat altered ruffled border and an increased number of vacuoles in the vicinity of the ruffled border,[78] thus suggesting an intracellular role played by the enzyme. A more direct clue for participation of TRAP in osteoclastic bone degradation was provided by Suter and colleagues.[79] These authors analysed mice that were deficient in either TRAP or lysosome acid phosphatase (LAP) or both enzymes. They reported a somewhat more severe osteopetrotic effect for the double knockout mice. Of interest was their finding that in the absence of activity of TRAP, osteopontin dephosphorylation was strongly inhibited. In the TRAP and TRAP/LAP deficient mice immunoreactive osteopontin accumulated not only in the ruffled border area, but also in lysosome-like structures of the osteoclast. The latter structures also contained mineral crystallites. Under normal conditions mineral is not found in vacuoles of the osteoclast, since all mineral is dissolved extracellularly in the ruffled border area. The intracellular localization of crystallites in conjunction with osteopontin in the TRAP/LAP deficient mice suggests that extracellular demineralisation was disturbed. A plausible explanation is that, due to ineffective dephosphorylation of proteins like osteopontin by the acid phosphatases, part of the mineral was shielded from dissolution. Although it is still a matter of debate whether TRAP is secreted in the extracellular space of the ruffled border area,[80] its role in intracellular digestion of certain bone matrix components has become clear.[42] Alternatively (or in addition) TRAP may play a role in bone degradation by generating reactive oxygen species (ROS). ROS are capable of destroying organic bone matrix components and may thus contribute to bone resorption.[81]

Matrix Metalloproteinases

The sequence of degradative processes in the resorption zone is thought to keep pace with local pH development. When (during the initial stages) pH is low, not only demineralisation of bone proceeds effectively but also lysosomal enzymes like cysteine proteinases (and TRAP?) can act under favourable conditions. In addition to these enzymes several data suggest that also other proteinases participate in the digestion of bone matrix by osteoclasts. In particular matrix metalloproteinases are considered in this context.[82] However, the latter enzymes usually exert their activity at a somewhat higher pH (around pH 7). How to explain participation of both classes of enzymes? A possible explanation may be found in the sequence of activities occurring during resorption. At the onset of resorption the osteoclast lowers pH and thus will demineralise the bone matrix. Concomitantly cysteine proteinases are released and, due to the low pH, will digest part of the bone matrix, in particular noncollagenous proteins. This demineralisation/digestion results in the release of Ca^{2+} and protein fragments, which may cause local pH to increase somewhat,[83] sufficient to create an environment suitable for metalloproteinases to exert their activity.[72]

Metalloproteinases are suggested to be involved in the final degradation of the collagenous matrix.[72] However, the nature of the metalloproteinases involved has not been elucidated yet.[82] Several studies have shown the presence of one or more members of this group of enzymes in or around osteoclasts (reviewed in ref. 82). Of these, in particular MMP-9 appears to be produced by osteoclasts.[84] This enzyme, however, is probably not essential for the actual resorption but more important in migration of these cells.[85] An alternative -indirect- role has been proposed by Holliday et al,[86] who suggested that metalloproteinase-mediated collagen fragments stimulated osteoclastic bone degradation by triggering cytoskeletal reorganization and V-ATPase transport to the ruffled border.

Although most studies support the view that metalloproteinases participate at some stage in the sequence of events leading to bone resorption, a number of studies shed doubt on the role of these enzymes in the actual osteoclast-mediated resorption of matrix.[82] By using selective inhibitors of this class of enzymes, some authors did find an inhibiting effect,[49,72] whereas others were unable to note such an effect.[87] Since the different research groups made use of different bone systems (skull versus long bone) to analyse a contribution of metalloproteinases in osteoclastic bone degradation, a direct comparison of both systems was made. This study revealed differences in the enzymes employed by the different osteoclasts in the digestion of bone matrix.[88] Skull osteoclasts proved to use both cysteine proteinases and metalloproteinases in the digestion of bone matrix, whereas bone digestion by osteoclasts of long bone was mediated primarily by cysteine proteinases. These data strongly suggest site-specific differences between osteoclasts. Whether in addition to the two different populations of osteoclasts other populations exist needs further clarification. In this respect is the finding of Fox et al[89] of interest who noted differences in the response to Il-1 between native osteoclasts and osteoclasts generated in vitro. This may suggest that osteoclasts active during normal remodelling of bone differ from those active under other—pathologic(?)—conditions and that different subsets of osteoclasts are differently modulated in their activity. Whether this is due to differences in monocyte subsets that generate the osteoclasts,[88] needs further clarification.

Concluding Remarks

During the last decade several studies have appeared presenting crucial information not only on the processes involved in the formation of osteoclasts,[10,90] but also on the sequence of events leading to resorption of mineralised tissues by these cells.[11,82] It has become apparent that the osteoclast is a highly polarized cell,[25,26] with a series of unique specialized features. These cells create an external environment (ruffled border area) in which the pH is lowered and a variety of proteolytic enzymes are secreted. In the acidic milieu of the ruffled border area the mineralised tissue is completely demineralised, which is followed by (partial) digestion of matrix proteins. Subsequent endocytosis of matrix fragments is followed by further digestion in the lysosomal system. Remnants are released in the extracellular space after transcytosis (Fig. 5).

Acknowledgements

We thank Jenneke Klein-Nulend for helpful comments on the manuscript.

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CHAPTER 13

Membrane Resealing Mediated by Lysosomal Exocytosis

Norma W. Andrews*

Abstract

Ca^{2+}-regulated exocytosis was proposed to mediate plasma membrane repair, but until recently the nature of the vesicles involved was unknown. Recent work from our laboratory identified lysosomes as the intracellular organelles responsible for this process. Ca^{2+} triggers the exocytosis of conventional lysosomes, and this process is regulated by a ubiquitously expressed synaptotagmin, Syt VII. Dominant-negative or gene deletion approaches revealed that Syt VII is required for normal lysosomal exocytosis and membrane resealing. Cells from Syt VII-deficient mice show defects in lysosomal exocytosis and membrane repair, and the animals develop a form of autoimmune myopathy similar to the human diseases polymyositis/dermatomyositis.

Secretion of lysosomal enzymes is a common physiological event, often observed in a variety of tissues.[1,2]L ysosomes, however, have been traditionally regarded as "terminal" compartments, the final site of accumulation of endocytosed macromolecules.[3,4] This concept is derived mostly from the fact that lysosomes do not constitutively recycle to the plasma membrane, in contrast to what is observed with early endosomes. The norm thus became to interpret lysosomal enzyme secretion as a result of altered sorting pathways: the enzymes would be diverted at the level of the trans-Golgi network into "classical" Ca^{2+}-regulated secretory granules.[2,5] However, in several cases there is no direct evidence that lysosomal enzymes colocalize with "classical" secretory products inside specialized granules.[6,7] And as further discussed in this chapter, many cell types that lack "classical" secretory granules secrete lysosomal enzymes when stimulated. Therefore, it is conceivable that a significant fraction of the observations previously interpreted as alterations in lysosomal enzyme sorting are a consequence of direct exocytosis of conventional lysosomes. In "professional" regulated secretory cells, conventional lysosomes may be secreted in parallel with "classical" granules, since the $[Ca^{2+}]_i$ elevation induced by a secretagogue can trigger both processes (Fig. 1). We discuss below recent evidence suggesting that this pathway of conventional lysosome exocytosis has an important role in plasma membrane repair.

"Secretory" Lysosomes

Several specialized secretory cells contain regulated secretory granules that are clearly distinct from lysosomes, such as the zymogen granules of pancreatic acinar cells.[8] However, it is interesting to note that a significant number of regulated secretory compartments from different cell types have many properties in common with lysosomes. The most widely recognized

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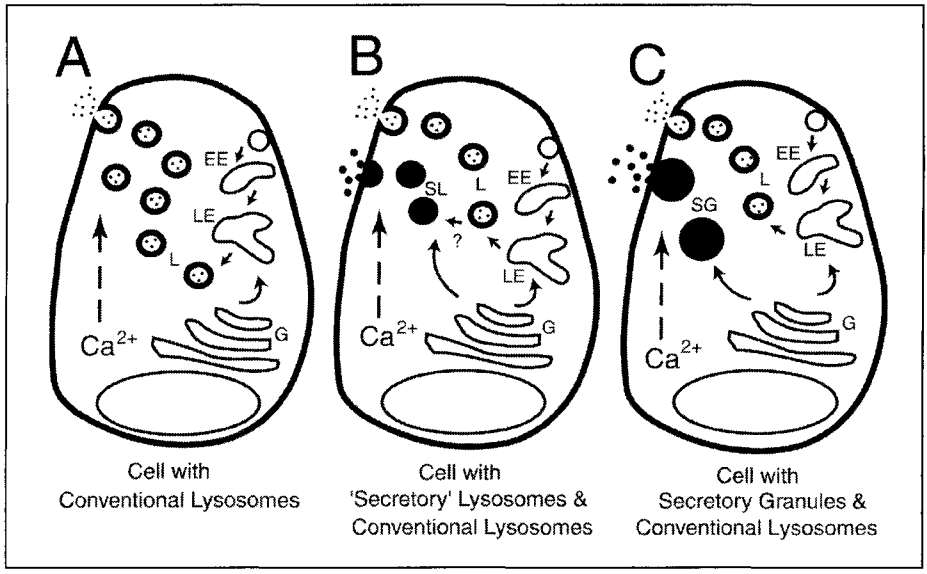

Figure 1. Ca^{2+}-regulated exocytic compartments in mammalian cells. A) Nonspecialized secretory cell. B) Cell that secretes specific products from granules with lysosomal properties (in CTLs, Ls and SLs largely overlap). C) Cell that secretes specific products from granules distinct from lysosomes. EE, early endosomes; LE, late endosomes; L, conventional lysosomes; SL, "secretory" lysosomes; SG, secretory granules; G, Golgi. Low μmolar levels of $[Ca^{2+}]_i$ are sufficient for triggering exocytosis in examples of the 3 cell types—fibroblasts,[67] mast cells[68] and pancreatic acinar cells.[69]

examples are found in hemopoietic cells: α-granules from platelets, azurophil granules from neutrophils, lytic granules from cytotoxic lymphocytes and basophilic granules of mast cells have acidified lumens, and contain acidic hydrolases and lysosomal membrane markers. In most cases, these granules have been shown to be accessible through the endocytic pathway.[9-12] Osteoclasts (see Chapter 12), which also belong to the hemopoietic lineage, show a dramatic reorganization of the lysosomal compartment, with translocation of lysosomal glycoproteins to the ruffled border membrane and secretion of lysosomal enzymes at the site of bone resorption.[13] These granules, generally referred to as "secretory" lysosomes, were for a long time considered to be a specific adaptation present only in hemopoietic cells.[14]

Interestingly, however, there are several examples of cells with "secretory" lysosomes that do not belong to the hemopoietic lineage. Melanosomes, the melanin-containing granules transferred from melanocytes to keratinocytes, share a common biogenesis and several characteristics with lysosomes.[15] In pulmonary alveolar type II cells, the lysosome-like lamellar bodies are responsible for the Ca^{2+}-regulated secretion of surfactant.[16] The acrosome of mammalian spermatozoa, another Ca^{2+}-regulated exocytic compartment, has also been considered to be a modified lysosome, owing to its acidic lumen containing a full set of acidic hydrolases.[17] And recent observations revealed an intriguing overlap between markers for lysosomes and Weibel-Palade bodies, the regulated secretory granules of endothelial cells.[18] In response to injury, endothelial cells are activated by inflamatory mediators such as thrombin or histamine (which elevate $[Ca^{2+}]_i$), triggering exocytosis of Weibel-Palade bodies and release of von Willebrand factor. Interestingly, von Willebrand factor, an adhesive protein involved in primary hemostasis, is also secreted by the lysosome-like alpha-granules of megakayocytes and platelets.[19] Therefore, it is clear that a capacity for Ca^{2+}-regulated exocytosis is commonly associated with lysosomal properties, and that this occurs independently of cell lineage.

Ca^{2+}-Regulated Secretion of Conventional Lysosomes

Our interest in investigating the capacity of conventional lysosomes to behave as Ca^{2+}-regulated exocytic vesicles was born from our studies of the cell invasion mechanism of the protozoan parasite *Trypanosoma cruzi*. During these studies, we were surprised to observe a striking recruitment and clustering of lysosomes at the parasite attachment site, in a variety of cell types. As trypanosomes entered the cell, lysosomes gradually fused with the plasma membrane, in what resembled an exocytic process. However, instead of lysosomal contents being released extracellularly, a tight seal was formed between the parasite and the lumen of lysosomes, and the parasite eventually entered the cell in a compartment formed by lysosomal membranes.[20,21] This process was found to be dependent on intracellular Ca^{2+} transients triggered in host cells by the parasites.[22-26] Since *T. cruzi* invades a large variety of mammalian cells by this unusual lysosome-dependent mechanism, these observations suggested that conventional lysosomes were able to respond to $[Ca^{2+}]_i$ elevations by fusing with the plasma membrane. Evidence supporting this hypothesis was obtained in a series of subsequent studies, by our group and other investigators. These studies showed that the *T. cruzi* cell invasion mechanism has a large number of properties in common with Ca^{2+}-regulated exocytosis of lysosomes.[21,27,28]

Regulated secretion has been generally considered to be a highly specialized cellular process. Surprisingly, several recent studies detected a large component of Ca^{2+}-regulated exocytosis in cells previously believed to be only capable of constitutive secretion, such as fibroblasts and epithelial cells.[29-31] Membrane capacitance measurements revealed a 20-30% increase in the surface area of CHO and 3T3 fibroblasts after elevation in $[Ca^{2+}]_i$.[30] In CHO cells, the diameter of the Ca^{2+}-regulated exocytic vesicles detected by capacitance measurements was estimated to be between 0.4 and 1.5 μm in diameter.[31] This is a large size, very consistent with the dimensions of lysosomes in these cell types.[32] Interestingly, even in cells such as PC-12 and adrenal chromaffin cells, which contain "classical" Ca^{2+}-regulated secretory granules, an additional population of large exocytic vesicles with distinct properties was detected by electrophysiological methods.[33,34] Such studies reinforced the growing perception that most cell types contain a population of vesicles that can be mobilized for fusion with the plasma membrane upon elevation in $[Ca^{2+}]_i$ .Detailed studies performed with NRK cells in our laboratory identified these vesicles as conventional lysosomes.[35] Upon stimulation with 1 μM Ca^{2+}, the lumenal domain of lysosomal membrane glycoproteins is exposed on the plasma membrane, and lysosomal contents are released extracellularly. In contrast, no significant increase in cell surface recycling of early endosomes is observed, under the same conditions. Furthermore, only the lysosomally-processed form of cathepsin D is secreted in response to $Ca,^{2+}$ reinforcing the conclusion that mature lysosomes, and not biosynthetic carrier vesicles, are involved in this exocytic process.[35]

Regulation of Lysosomal Exocytosis by Synaptotagmin VII

Members of the synaptotagmin family of Ca^{2+}-binding proteins have been proposed to have a role as Ca^{2+} sensors in the regulation of exocytosis, in neurons and other cell types. Synaptotagmins are transmembrane proteins with a short amino terminus ectodomain, a single transmembrane region and two highly conserved, independently folding Ca^{2+}-binding C_2 domains (C_2A and C_2B) homologous to the C_2 regulatory region of protein kinase C[36,37] (Fig. 2).

Synaptotagmin I (Syt I), the most extensively studied isoform, is present on the membrane of synaptic vesicles in neurons. Genetic studies in mice, *Drosophila* and *C. elegans* demonstrated that Syt I ablation or mutation strongly decreases the Ca^{2+} dependency of neurotransmitter release.[38-40] Several of the rat and mouse synaptotagmin isoforms described to date are primarily found in brain, but, interestingly, some are also expressed in other tissues.[41-43]

Syt VII, in particular, was shown to be expressed at significant levels on most mouse tissues.[41-44] Consistent with this ubiquitous pattern of expression, recent work from our laboratory showed that Syt VII is localized on the membrane of lysosomes in NRK[45] and other cell types.[28] Isoform-specific, affinity-purified antibodies detected Syt VII on lysosomes in immunofluorescence assays,[28,45] and in sub-cellular fractionations followed by western blot.[45] In

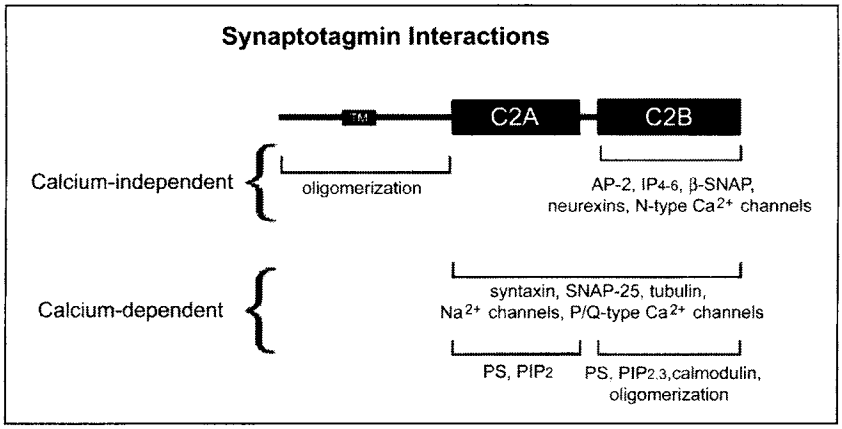

Figure 2. Structure of Synaptotagmin I indicating the Ca^{2+}-dependent and independent interactions/functions.[70] C_2A and C_2B indicate the Ca^{2+}-binding cytosolic domains; TM indicates the transmembrane domain.

NRK cells, no colocalization was observed with the early endosomal markers EEA1 and transferrin, or the late endosomal marker Rab 7. Furthermore, GFP-tagged full-length Syt VII is targeted to lysosomes when transfected into NRK[45] (Fig. 3*J*) and other cell types.

Recently proposed models for the role of Syt I in synaptic vesicle exocytosis suggest that Ca^{2+}-triggered interactions involving the C_2 domains alter the physical relationship between the SNARE complex and lipid bilayers, regulating fusion.[46] The C_2A domains of Syt I and of several additional isoforms were shown to interact with syntaxin 1 and with acidic phospholipids in a Ca^{2+}-dependent manner[41] (Fig. 2), and antibodies generated against the Syt I C_2A domain, and recombinant peptides containing the Syt I C_2A domain, inhibit Ca^{2+}-triggered exocytosis when introduced into neuronal cells.[47-49] Similarly, we found that the C_2A domain of Syt VII or antibodies against this domain block Ca^{2+}-triggered exocytosis of lysosomes in NRK cells and human fibroblasts.[45] Inhibition of lysosomal exocytosis was only observed in the presence of the Syt VII C_2A domain, and not the C_2A domain of the exclusively neuronal isoform Syt I. Expression of the C_2A domain of Syt VII also inhibited cell invasion by *T. cruzi*, consistent with our previous evidence indicating that this parasite subverts the Ca^{2+}-regulated lysosomal exocytic pathway for establishing intracellular infections.[28] These observations suggested that Syt VII functions as a specific, high-affinity regulator of Ca^{2+}-triggered lysosomal exocytosis.

Role of Ca^{2+}-Regulated Exocytosis of Lysosomes in Plasma Membrane Resealing

A series of studies in the last decade concluded that plasma membrane repair in animal cells involves the delivery of intracellular membrane to wound sites, by a mechanism resembling Ca^{2+}-regulated exocytosis. Ca^{2+} influx through plasma membrane disruptions triggers a high rate of vesicular exocytosis at the wound site, an event that is required for membrane resealing.[50-52] Intracellular membrane delivery reduces plasma membrane tension,[53] leading to the suggestion that this may be an important component of the mechanism by which exocytosis promotes resealing.[54,55] These studies strongly suggested that a ubiquitous form of Ca^{2+}-regulated exocytosis is a necessary and rate-limiting step in plasma membrane repair. However, the exact nature of the intracellular vesicles involved in this important process remained obscure. In sea urchin eggs, resealing of plasma membrane disruptions was initially proposed to be mediated by cortical granules,[51] but in subsequent studies yolk granules were implicated.[56] In endothelial cells and fibroblasts, the intracellular compartment mobilized for exocytosis during wounding

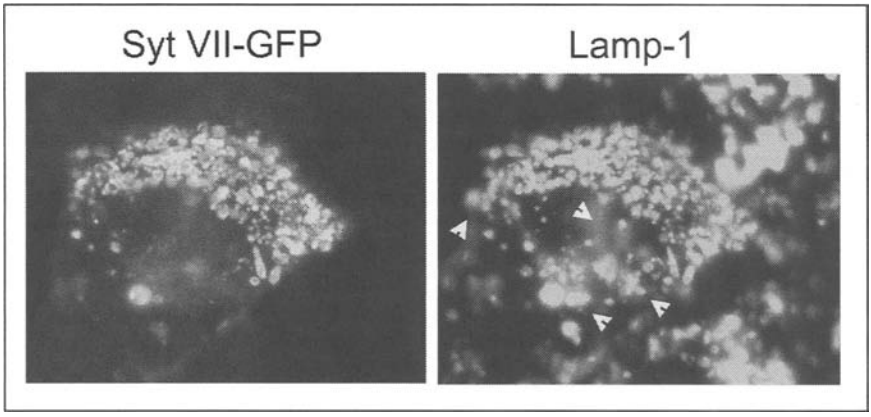

Figure 3. Targeting of GFP-tagged Synaptotagmin VII to lysosomes in NRK cells. NRK cells were transfected with Syt VII-GFP as previously described (ref Martinez). Left panel, GFP fluorescence; right panel, immunofluorescence with monoclonal antibodies against the lysosomal protein Lamp-1. There is extensive overlap between the localization of Syt VII-GFP and the anti-Lamp-1 fluorescence; the arrowheads point to the few compartments positive for Lamp-1 that do not contain Syt VII-GFP.

was proposed to be endosomal/lysosomal, since it was labeled by endocytosed FM-143. However, the very long continuous periods of dye uptake utilized (12 hours) did not allow a more precise identification of the vesicle population involved.[52-54] Based on an inhibition of resealing observed after brefeldin A treatment, vesicles derived from the Golgi apparatus were also proposed to have a role.[54] However, it is important to note that brefeldin A also affects the morphology and functional properties of lysosomes.[57]

Our detection of Ca^{2+}-regulated exocytosis of conventional lysosomes in many cell types suggested to us that this process might be involved in plasma membrane resealing. In a recent study, compelling evidence in support of this view was generated.[58] First, we showed that a lumenal epitope of the lysosomal glycoprotein Lamp-1 appears on the cell surface at the site of plasma membrane disruption, a process strictly dependent on the presence of extracellular Ca^{2+}. Second, we showed that the lumenal domain of Syt VII is also exposed on the cell surface shortly after wounding. Third, we found that soluble Syt VII C_2A domain recombinant peptides or antibodies against the Syt VII C_2A domain, agents that block lysosomal exocytosis, inhibit surface translocation of Lamp-1 and plasma membrane repair. Surface exposure of Lamp-1 was quantitated by immunofluorescence and flow cytometry, after cells were wounded by scraping. Plasma membrane repair was assessed in a two-tracer uptake assay: monolayers were wounded by contact with glass beads in the presence of Texas red-conjugated dextran, followed by rapid washes and transfer to BODIPY-conjugated dextran. All cells with plasma membrane lesions took in the first dye; uptake of the second dye occurred only when the wounds were not repaired. And fourth, we directly demonstrated a role for lysosomes in the resealing of microinjection wounds, by imaging Ca^{2+} influx after microinjection of antibodies against the C_2A domain of Syt VII, or against the cytosolic tail of Lamp-1. In contrast to the very transient rise in $[Ca^{2+}]_i$ observed after microinjection of control IgG, a significantly sharper and sustained $[Ca^{2+}]_i$ elevation was observed after microinjection of affinity-purified anti-Syt VII C_2A antibodies, an event that was followed by detachment of the cells from the coverslip. Importantly, a similar pattern was observed after microinjection of affinity-purified antibodies against the cytosolic tail of Lamp-1.[58] As previously demonstrated in our laboratory, these antibodies specifically aggregate lysosomes when microinjected into several cell types.[21,58,59] Therefore, through inhibition of Syt VII function or by additional Syt VII-independent approaches, strong evidence supporting the involvement of lysosomes in plasma membrane repair was obtained.

A Model for the Study of Plasma Membrane Wounding and Repair under Physiological Conditions

The findings described above linking Ca^{2+}-regulated exocytosis of lysosomes to the repair of plasma membrane lesions were based on observations made on mechanically-wounded cultured cell lines. We were interested in verifying if the same process was responsible for the resealing of primary cells, wounded under physiological conditions. Previous studies in rodents showed that cells from tissues such as skin, gut and muscle, which are routinely subjected to mechanical stress, are frequently injured (as concluded from the incorporation of membrane-impermeant molecules into their cytosol).[60-62] Interestingly, the frequency of wounding in rat skeletal muscle cells was reported to increase proportionally with exercise.[61] Primary skin fibroblasts, when locomoting in culture, also suffer frequent and rapidly reversible plasma membrane ruptures, during retraction of the trailing edge.[63] Such lesions, proposed to be caused by rupture of the focal adhesions formed between fibroblasts and the substrate, also occur extensively in the fibroblast-collagen-matrix model of wound contraction.[64] This is a well-established model that has been widely used to study the regulation of wound contraction, a critical step in the healing of cutaneous lesions.[65,66] In this system, skin fibroblasts embedded in a three dimensional matrix of polymerized collagen attached to a substrate (an environment that mimics the granulation tissue secreted by fibroblasts in cutaneous wounds) develop extensive stress fibers and strong focal adhesions. The tension generated by the fibroblasts under these conditions is considered to be equivalent to the force responsible for contraction and closure of cutaneous wounds in vivo. This contraction event is reproduced in an accelerated scale when collagen-embedded fibroblast matrices are detached from the substrate. After release, the floating matrix condenses in a few seconds into a dense body one tenth of its original size, as a result of the isometric tension generated by the fibroblasts. Work performed in the laboratory of Fred Grinnell demonstrated that during this contraction event the majority of the matrix fibroblasts become permeable to extracellular dextran, due to plasma membrane rupture at focal adhesion sites. These lesions are resealed in a few seconds in a Ca^{2+}-dependent fashion, and the fibroblasts can be extracted from the matrix and recultured, retaining full viability.[64] This system provided us with a very sensitive and quantitative assay for the study of lysosome-mediated plasma membrane repair in primary cells.

In this procedure, human foreskin fibroblasts are ressuspended in a collagen solution, which is then added to plastic dishes and allowed to polymerize, as a "drop" attached to the substrate. After polymerization, the three-dimensional fibroblast-collagen matrices is either left attached or lifted from the dish with a spatula in the presence of membrane-impermeable, lysine-fixable Texas red-dextran, washed, fixed and prepared for confocal microscopy. As discussed above, fibroblasts in anchored matrices show a highly branched and extended morphology, with abundant actin stress fibers evident after fluorescein-phalloidin staining, and no Texas red-dextran in their cytosol.[58,64] Upon release from the substrate, the matrix fibroblasts undergo a synchronized contraction, visualized after fixation as shortened cell bodies with greatly reduced stress fibers. This contraction event causes extensive plasma membrane wounding, detected through the cytosolic incorporation of Texas red-dextran. When these anchored and released matrix fibroblasts were examined for the presence of surface-exposed Lamp-1, the results were the same as those obtained with mechanically-wounded cell lines: only Texas red-dextran-positive, wounded fibroblasts had detectable fluorescence after surface staining with mAbs to the lumenal domain of human Lamp-1.[58]

More than 90% of the matrix fibroblasts suffer plasma membrane wounds as they contract their supporting collagen matrix.[64] These lesions are, however, rapidly repaired in a strictly Ca^{2+}-dependent fashion, with the fibroblasts becoming again impermeable to extracellular tracers in a few seconds.[64] Such synchronous wounding of the majority of the cell population followed by rapid repair allows precise quantification of the process of plasma membrane repair. Lactate dehydrogenase (LDH) activity can be used to quantify cytosol leakage from wounded cells,

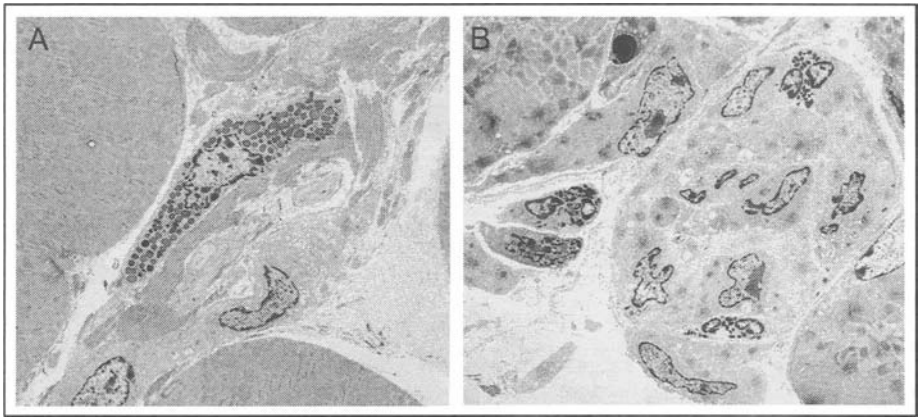

Figure 4. Inflammatory infiltration and collagen fiber deposition in the skeletal muscle of Syt VII-deficient mice. A) Extensive accumulation of collagen fibers next to a mast cell in the skeletal muscle endomysial space. B) A muscle fiber completely invaded by macrophages and eosinophils. Both images were obtained by transmission electron microscopy of quadriceps muscle of Syt VII-deficient mice.

and released β-hexosaminidase activity can be simultaneously measured as a marker for lysosomal exocytosis.

Another advantage of this system is that the viability of fibroblasts embedded in collagen matrices can also be precisely quantified. After the collagen matrices are released from the dish, undergoing contraction and wounding, the cells can be recovered by treatment with collagenase and trypsin, recultured for 24 h, and their viability assessed by ^{3}H thymidine incorporation. Our findings using this system indicated that, whereas the continuous presence of Syt I C_2A had no effect, a significantly smaller number of viable fibroblasts is extracted from matrices kept in the presence of Syt VII C_2A, a result consistent with a deficiency in the ability to reseal the plasma membrane.[58]

Defective Membrane Resealing and Inflammatory Myopathy in Syt VII-Deficient Mice

Having learned that Syt VII-regulated lysosomal exocytosis appears to play a central role in the repair of plasma membrane wounds, we became interested in determining what would be the consequence of inhibiting this process in vivo. To this end, Syt VII-deficient mice were generated.[67] These animals are viable, but exhibit a reduced level of activity that is apparent a few weeks after birth. To determine if defects in plasma membrane repair could be involved in this phenotype, fibroblast collagen matrix contraction experiments were performed using embryonic fibroblasts. Reduced release of β-hexosaminidase and an altered, prolonged pattern of LDH release were observed, consistent with defects in lysosomal exocytosis and plasma membrane resealing.[67]

Further histological analysis of the mouse tissues was very informative, and provided an explanation for the reduced level of activity of Syt VII-deficient mice: A marked inflammatory process with muscle fiber destruction was observed in skeletal muscle. This inflammatory process had an early onset, with large numbers of infiltrating cells being detected in skeletal muscle by 4 weeks of age. At 8 weeks, extensive endomysial deposition of collagen fibers was evident, with accumulation of mast cells (Fig. 4A) and invasion of muscle fibers by macrophages and eosinophils (Fig. 4B). Collagen accumulation was also detected in the skin, but not in other organs,[67] suggesting that the phenotype might be restricted to tissues under mechanical stress. Interestingly, inflammatory myopathy, with foci of muscle fiber

degeneration followed by progressive muscle weakness (which also develops in older Syt VII-deficient mice), strongly resemble the symptoms associated with the severe human autoimmune diseases polymyositis/ dermatomyositis. Remarkably, Syt VII-deficient mice also develop anti-nuclear antibodies, which is a hallmark of autoimmune disorders. These findings raise the intriguing possibility that defective membrane repair in tissues under mechanical stress, with the consequent release of intracellular antigens, may favor the development of autoimmunity.

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CHAPTER 14

Macroautophagy in Mammalian Cells

Eeva-Liisa Eskelinen*

Abstract

Autophagy is a lysosomal degradation pathway for cytoplasmic material, which is activated during stress conditions such as amino acid starvation or viral infection. Yeast mutants defective in autophagy do not survive starvation. Mammalian cells use autophagy during short periods of starvation to degrade nonessential cellular components in order to liberate nutrients for vital biosynthetic reactions. Recent results have shown that autophagy also contributes to development, growth regulation and cancer, as well as longevity. Although the autophagic pathway was described several decades ago, the first molecular mechanisms have only been recently revealed due to the discovery of autophagy-defective yeast mutants.

Depending on the delivery route of the cytoplasmic material to the lysosomal lumen, three different autophagic routes are known: 1) macroautophagy, or simply autophagy, 2) microautophagy (Fig. 1), and 3) chaperone-mediated autophagy. In macroautophagy, a portion of cytoplasm to be degraded is first wrapped inside a specialised organelle, the autophagosome, which then fuses with lysosomal vesicles and delivers the engulfed cytoplasm for degradation[1] (Fig. 2). In microautophagy, the lysosomal membrane itself sequesters a portion of cytoplasm by a process that resembles pinching off of phagosomes or pinosomes from the plasma membrane.[2] Macroautophagic uptake of cytoplasmic material appears to be a nonselective process;[3] organelles are sequestered at the same frequency as they exist in the cytoplasm. In chaperone-mediated autophagy, proteins possessing a specific sequence signal are transported from the cytoplasm, through the lysosomal membrane, to the lysosomal lumen[4] (Chapter 15). A fourth autophagic route, crinophagy, has also been described.[5] In crinophagy, secretory vesicles directly fuse with lysosomes, which leads to degradation of the granule contents. This review concentrates on the (macro)autophagic pathway in mammalian cells.

Macroautophagic Pathway

After induction by a stress signal such as amino acid starvation, the first step in autophagy is the formation of an *autophagosome* (Figs. 2, 3A). A flat membrane cistern elongates and wraps itself around a portion of cytoplasm, forming a double-membrane bound autophagosome. Autophagosomes next receive lysosomal constituents, such as lysosomal membrane proteins and proton pumps, from endosomal vesicles via vesicle-mediated transport. Finally the outer limiting membrane of the autophagosome fuses with the limiting membrane of a late endosome, multivesicular body (MVB), or lysosome[6-12] (Figs. 2, 3C). In this process the cytoplasm, still engulfed by the inner limiting membrane, is delivered to the endo/lysosomal lumen (Figs. 2, 3D). Both the cytoplasm and the membrane around it are then degraded by lysosomal hydrolases, and the degradation products are transported back to cytoplasm where they can be reused for metabolism. By definition, autophagosomes, also called *initial autophagic vacuoles*

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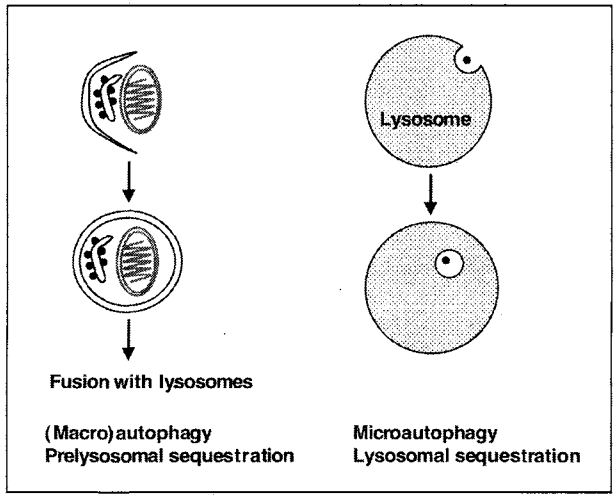

Figure 1. The different ways of sequestration of cytoplasmic material in macro- and microautophagy. See text for further details.

(AVi), do not yet contain lysosomal membrane proteins or enzymes, and they are not acidic[13] (Figs. 4A, 5). During the maturation process autophagosomes develop into *late*, or *degradative autophagic vacuoles (AVd)*, which are acidic and contain lysosomal membrane proteins and enzymes[10] (Fig. 4B,C). The latter have also been called *autolysosomes*. Quantitative immunoelectron microscopy has been used to demonstrate the enrichment of lysosomal membrane proteins and enzymes in late autophagic vacuoles.[14,15] The lack of integral membrane proteins in autophagosomes was first revealed by freeze-fracture electron microscopy. Other

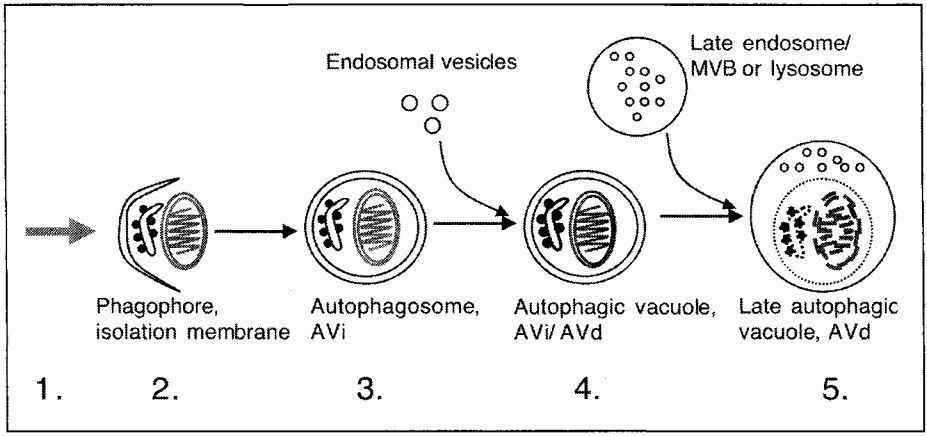

Figure 2. The macroautophagic pathway is induced by a stimulus (1) such as amino acid starvation. After induction, a flat membrane sack wraps around a portion of cytoplasm (2) and finally forms a closed structure, autophagosome (3), which has two limiting membranes. Vesicular traffic from endosomal compartments delivers lysosomal membrane proteins to autophagosomes (4). Autophagosomes finally fuse with late endosomes, multivesicular bodies (MVB), or lysosomes, (5) delivering the sequestered cytoplasm, still surrounded by the inner limiting membrane, to the endo/lysosomal lumen. Both the remaining limiting membrane and cytoplasm are then degraded and the degradation products transported back to cytoplasm.

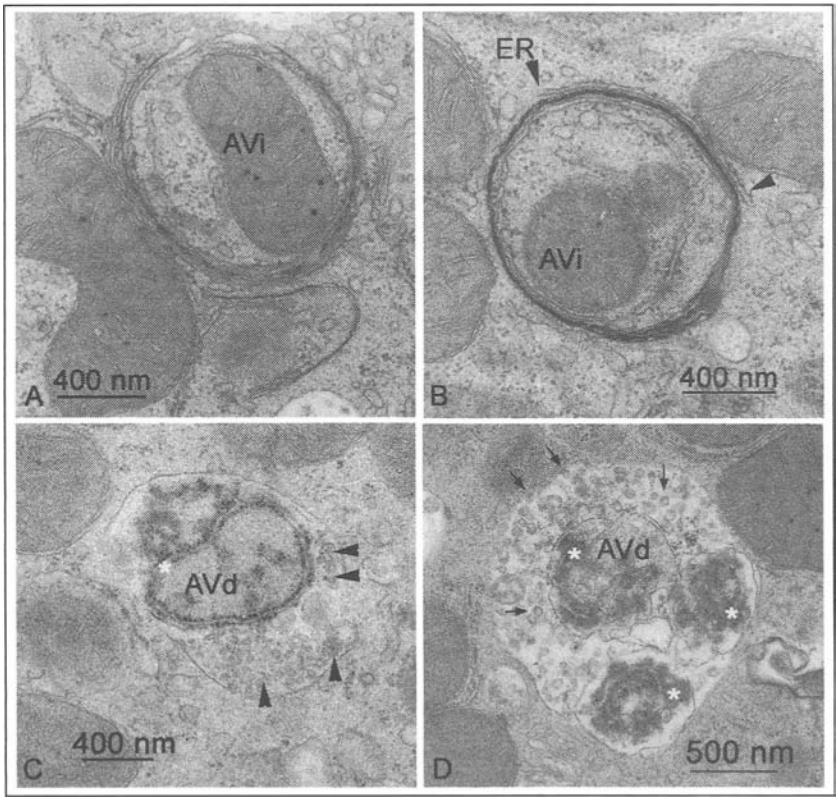

Figure 3. Morphology of autophagosomes and autophagic vacuoles in isolated mouse hepatocytes. A) An autophagosome, or initial autophagic vacuole (AVi), containing a mitochondrion, endoplasmic reticulum membranes, and ribosomes. The two limiting membranes of the autophagosome are visible at the upper rim of the vacuole. Below the AVi a flat membrane cistern seems to be in the process of sequestrating a peroxisome. B) Autophagosome membranes are often sandwiched between two cisterns of rough endoplasmic reticulum (ER). Arrowheads point the ER outside the AVi, and another ER cistern is present inside the limiting membrane just opposite. C) The contents of this late/degradative autophagic vacuole (AVd) look partially degraded, but the remnants of rough ER can still be identified (indicated by asterisk). The cell was fed with 6-nm gold particles coated with albumin for 2 h before fixation. As a result of fusion with a multivesicular endosome, the AVd contains both 6-nm gold particles (arrowheads) and small vesicles (round structures surrounding the gold particles). D) The degradation of the engulfed rough ER is advanced in this AVd, the remnants of ribosomes form electron-dense partially amorphous masses (indicated by asterisks). Also this AV has fused with a multivesicular endosome, as indicated by the content of numerous small vesicles (arrows).

cellular membranes such as lysosomal and endoplasmic reticulum membranes contain numerous integral membrane particles, considered to represent integral membrane protein molecules revealed by the freeze fracture procedure. However, the surfaces of the membranes limiting autophagosomes are almost completely smooth[16-18] (Fig. 5).

The origin of the membrane cistern forming new autophagosomes has been the subject of numerous studies, but still this issue is unresolved in mammalian cells. Many older ultrastructural studies suggested that smooth endoplasmic reticulum (ER) cisternae are the source of autophagosome membranes,[19] but evidence against this interpretation has also been published.[20] Recent studies in yeast have revealed that autophagosomes originate from a unique

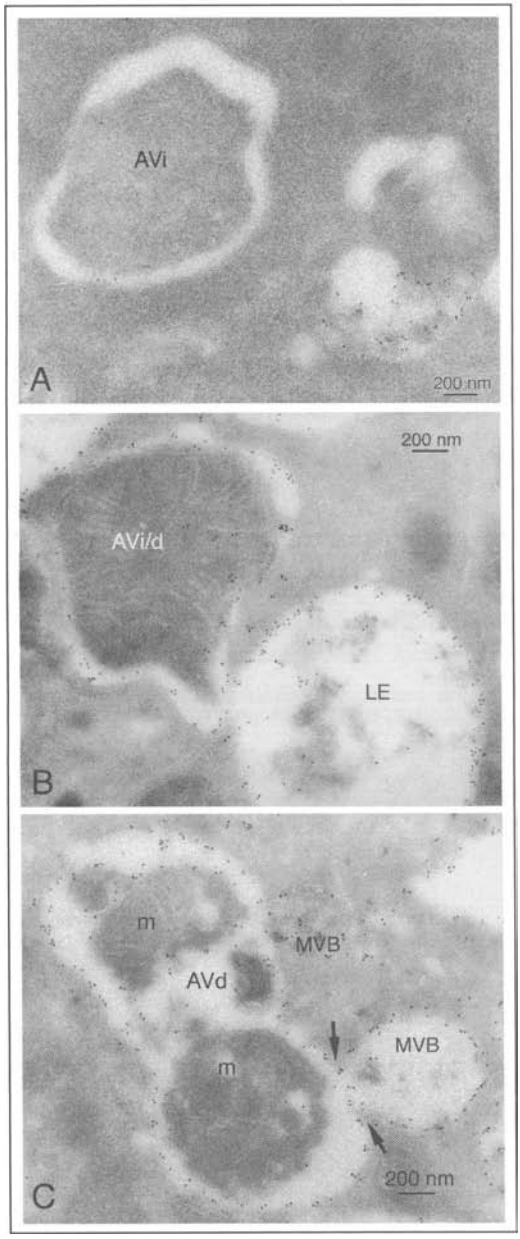

Figure 4. Localisation of the lysosomal membrane protein LAMP-1 in autophagic structures. The cryosections were prepared from isolated mouse hepatocytes and immunogold labelled using rat anti-LAMP-1, rabbit anti rat IgG, and goat anti-rabbit IgG coupled to 10-nm gold. A) A large proportion of autophagosomes (AVi) is devoid of LAMP-1 labelling. B) The autophagosome (AVi/d) has intense labelling for LAMP-1 in the limiting membrane, suggesting that it has fused with endo/lysosomal vesicles. LE, a late endosome. C) A fusion profile of two late autophagic vacuoles (AVd) and two multivesicular bodies (MVB). The autophagic vacuoles contain remnants of mitochondria (m). Arrows indicate the fusion pore between one of the MVBs and the AVd.

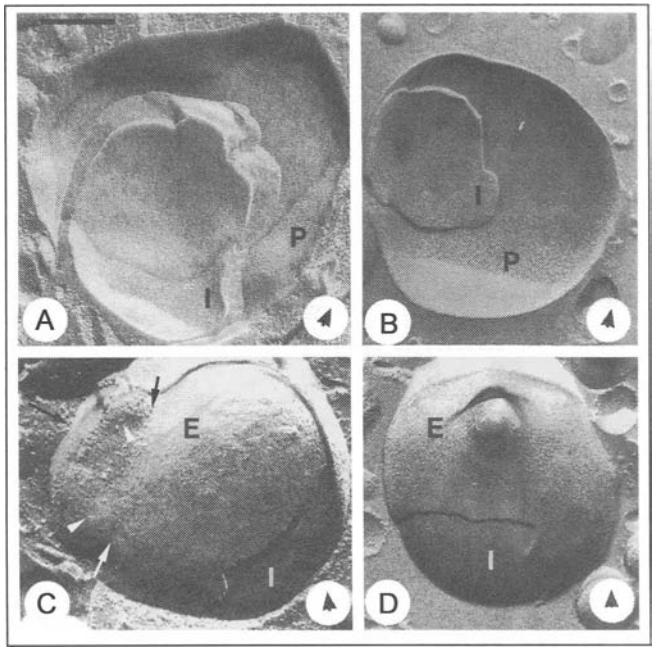

Figure 5. Freeze-fracture electron microscopy of autophagosomes from mouse hepatocytes. Panels A and C are from intact hepatocytes; B and I show isolated autophagosome fractions. Note that autophagosome membranes are almost completely free of intramembrane particles, putative integral membrane proteins. In panel C, arrows indicate a fusion event or the autophagosome with a smaller vesicles, putative endosome, which has intramembrane particles (arrowheads). P indicates the cytoplasmic fracture face of the outer limiting membrane; E is the extracytoplasmic fracture face. I indicates the inner limiting membrane. Bar, 200 nm. Reproduced, with permission, from: Punnonen EL, Pihakaski K, Mattila K, Lounatmaa K, and Hirsimäki P: Intramembrane particles and filipin labelling on the membranes of autophagic vacuoles and lysosomes in mouse liver. Cell Tissue Res 1989; 258: 269-276 (Fig. 1).

compartment called the preautophagosomal structure (PAS).[21,22] Because the autophagic pathway and genes involved in it are well conserved from yeast to mammals, it is likely that a similar unique compartment is the source of membrane in mammalian cells. Interestingly, ER cisternae are often observed on both sides of autophagosome membranes (Fig. 3B). Although in yeast membrane transport out of the ER seems to contribute to autophagosome formation,[23] in mammalian cells this does not seem to be the case.[24] Thus the possible function of the close ER contacts during autophagosome formation is still unknown.

The maturation of autophagosomes to late autophagic vacuoles is a relatively quick process. Both AVis and AVds are detected soon after the beginning of amino acid starvation. The half-life of autophagosomes has been estimated to be only 8 minutes.[25] When amino acid starvation is stopped and the cells are chased in full culture medium with fetal calf serum (FCS), the degradation and disappearance of the autophagic vacuoles can be followed by quantitative electron microscopy (Fig. 6A). Autophagosomes disappear first, while late autophagic vacuoles last longer. At the same time the volume fraction of endo/lysosomes undergoes a pulse-like increase reflecting the maturation of AVds to endo/lysosomes. It should be noted that the cells in Figure 6A were chased in the presence of 3-methyladenine, which may have prolonged the half-life of the AVds. When HeLa cells were first starved for amino acids for 2 h, and then chased in full medium for another 2h (without 3-methyladenine), both AVis and Avds had completely disappeared, indicating a faster consumption of AVds.

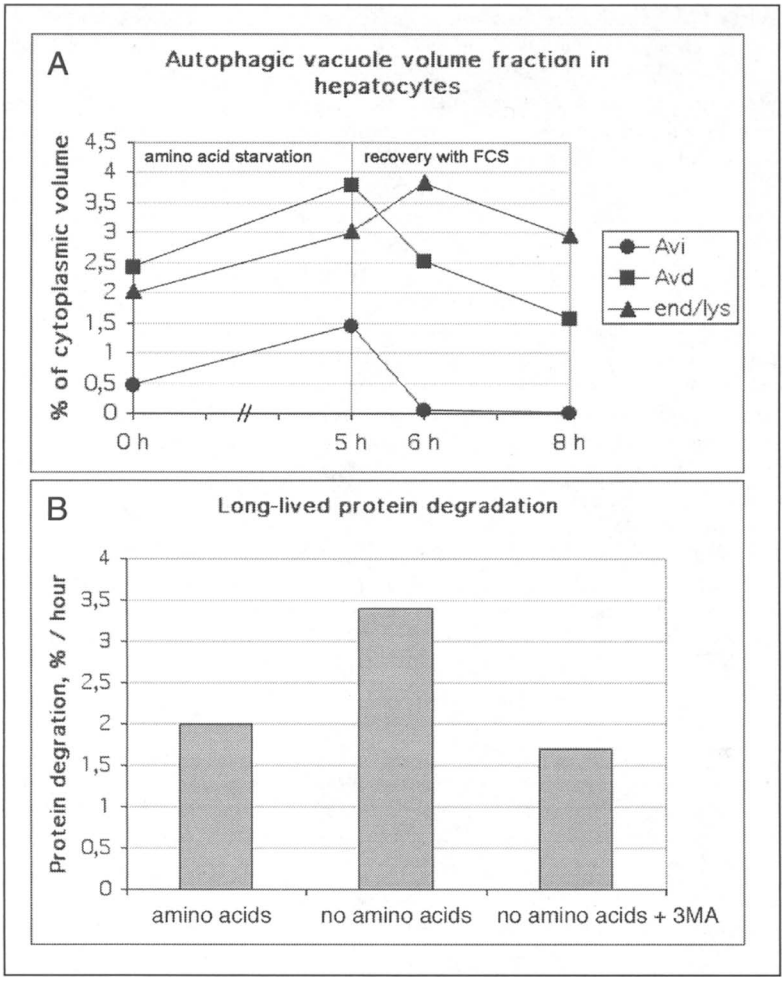

Figure 6. Quantitation of autophagy in isolated mouse hepatocytes. A) Quantitative electron microscopy was used to estimate the cytoplasmic volume fraction of autophagosomes (Avi) late autophagic vacuoles (Avd), and endo/lysosomes (end/lys) before treatment (0 h), after 5-h amino acid starvation (5 h), and 1 h and 3 h after finishing the starvation by replacing the starvation medium with full culture medium containing 3-methyladenine (6 h, 8 h). B) Degradation of long-lived proteins in hepatocytes incubated in the presence of full medium (amino acids), without amino acids, and without amino acids but in the presence of 3-methyladenine (3MA). Panel A is modified from reference 14 and panel B from reference 15.

Autophagosome formation is partially inhibited by drugs that disturb actin polymerisation,[26] suggesting that actin filaments may have a role in this process. Microtubule inhibitors like vinblastine and nocodazole retard maturation of autophagosomes, causing either AVis or AVds, or both, to accumulate.[26,27] It is possible that intact microtubules are needed for movement of autophagosomes and/or lysosomal vesicles, which in turn is necessary for fusion.

Induction and Regulation of Macroautophagy

The best known inducer of macroautophagy in mammalian cells is starvation of serum and amino acids. First autophagosomes can be detected already after 15-30 min of starvation.[28]

When measured by quantitative electron microscopy, the level of autophagic vacuoles reaches a steady state level in 2 – 4 h, and then it slowly starts to decrease.[29] During long-term starvation, chaperone-mediated autophagy increases and autophagy decreases.[30,31] This way the cell can prevent wasting too much of its cytoplasm via the nonselective macroautophagic pathway. In cultured cells, starvation of serum, but not amino acids, also increases the cytoplasmic volume fraction of autophagic vacuoles, but less than starvation of both serum and amino acids.[29] Viral infections, especially *Herpes simplex* virus, also may induce autophagy.[32]

The signal transduction pathways leading to autophagy activation during amino acid starvation have received attention lately. Two amino acid sensors have been described: the protein kinase Gcn2, which is activated by uncharged transfer RNA molecules,[32] and an amino acid receptor located on the plasma membrane.[33] The signals from Gcn2 to autophagy are mediated by a pathway including the eukaryotic initiation factor eIF2α, which supports autophagy when phosphorylated at Ser51.[32] eIF2α could thus act as a switch between protein synthesis (in the presence of amino acids) and autophagic protein degradation (in the absence of amino acids). The putative amino acid receptor at the plasma membrane seems to mediate the signals from an extracellular nontransportable leucine analogue to the autophagic pathway.[33] Future studies will show whether both suggested amino acid sensors are active in the same cells, and whether different amino acids use different signal transduction pathways to regulate autophagy and protein synthesis.

In yeast, the protein kinase TOR plays a crucial role in autophagy regulation. Active TOR kinase inhibits autophagy, and inhibition of the kinase activity by rapamycin activates autophagy.[34] In mammalian cells the role of mTOR in autophagy regulation is less clear, the induction of autophagy by rapamycin is weaker than that by amino acid starvation.[35] It is possible that some regulatory pathways, such as insulin, include mTOR, while others, such as amino acids, do not.[33] Phosphatidylinositol 3-kinases (PI3-kinases) also regulate autophagy. The activity of class III PI3-kinase Vps34 is absolutely required for autophagosome formation[29,36] and accordingly this process is completely inhibited by PI3-kinase inhibitors including 3-methyladenine (3MA),[37] wortmannin and LY294002,[38] as well as microinjection of inhibitory antibodies against Vps34.[29] Interestingly, class I PI3-kinases have an opposite effect, the activation of these kinases inhibits autophagy.[36] Since the activities of class I PI3-kinases promote cell growth and these kinases are upregulated in many cancers, it is possible that this signalling pathway might be linked to the role of autophagy in growth control. Recent extensive reviews on the signalling pathways controlling autophagy are available.[39,40]

Functions of Autophagy

Autophagy is a stress response. The role of autophagy as a survival mechanism during short-term amino acid starvation was discussed above. Induction of autophagy during *Herpes simplex* virus infections and localisation of viral particles inside autophagic vacuoles has been proposed to indicate that autophagy acts as a host-defence mechanism in the infected cells.[32] Interestingly, the *Herpes* virus virulence protein, ICP34.5, was shown to inhibit autophagy, suggesting that the virus has developed a way to prevent the autophagic defence of the host cell. Autophagy may also help cells to defend against some intracellular bacteria.[41]

In muscle and heart cells autophagy seems to have a special housekeeping role in turnover of cytoplasmic constituents including mitochondria. This is revealed by myopathy and cardiomyopathy in patients and mice possessing a defective autophagic degradation due to deficiency of the lysosomal membrane protein LAMP-2[15,42,43] (Chapter 4). In addition autophagy has a role in growth regulation, as suggested by decreased autophagy during growth of kidney after unilateral nephrectomy.[44] Further, impaired autophagy has been proposed to contribute to cancer development. Beclin 1, a mammalian homologue of the yeast autophagy gene Apg6/Vps30, is monoallelically deleted in a large proportion of human breast and ovarian cancers. Overexpression of beclin 1 in a breast cancer cell line increases autophagy and decreases the growth and tumorigenicity of these cells.[45] Further, mice homoallelically deleted of beclin 1 have less autophagy and

more tumors than control mice.[46,47] In addition beclin 1-/- mouse embryos, which are completely unable to perform autophagy, die around embryonic day 8,[47] suggesting that autophagy has important roles in early embryonic development. Finally, autophagy may also contribute to longevity. Long-term reduced caloric intake increases longevity in several species. Recent evidence suggests that increased autophagic turnover of cytoplasmic constituents including mitochondria may contribute to the longer life in the long-term dieting animals.[48] Further evidence that autophagy contributes to longevity has come from *C. elegans* mutants possessing a defective insulin receptor (daf2 mutant), which live longer than control worms. The increased lifetime of these mutant worms was recently shown to depend on a functional autophagic pathway.[49]

Autophagy also has roles in apoptosis, or programmed cell death. Type II programmed cell death, or autophagic cell death, has been described in mammary carcinoma cells.[50,51] The so-called death kinase and tumor suppressor, DAPk, may function in the signalling pathway that links autophagy to cell death.[52] In this process autophagy is needed for the cell death program. It is also possible that autophagy may protect cells against another type of apoptosis, type I programmed cell death.[53]

Autophagy Genes

Yeast genetics has immensely enlarged our knowledge of the molecular mechanisms involved in autophagy. Today, at least 27 autophagy-related yeast genes are known, and numerous reviews have been recently written on their functions.[54-56] Originally these genes were called Apg, Aut, or Cvt genes, but a consensus has now been reached, with all autophagy-related genes being described by a common name, Atg.[57] Several mammalian homologues of yeast autophagy genes have been identified (summarised in Table 1), and this has shown that the mechanisms of yeast autophagy are conserved in mammals. The first autophagosome marker protein, MAP LC3, was described recently.[28] This protein was originally identified as a microtubule associated protein and named 'microtubule-associated- protein-light-chain-3'. LC3 is a small 16-18 kDa protein that is soluble in nonstarved cells, but becomes peripherally membrane-associated during amino acid starvation (Fig. 7B,C). By immunoelectron microscopy LC3 has been shown to associate to the inner and outer limiting membranes of autophagosomes[28] (Fig. 7D). During maturation of autophagosomes to autolysosomes, LC3 seems to dissociate from the outer limiting membrane.[28] In Western blots, two forms of LC3 are seen, LC3I and LC3II (Fig. 7A). After cell homogenisation and differential centrifugation, LC3I is found in the soluble fraction, and LC3II in the pelletable membrane fraction.[28] In yeast, and probably also in mammals, the membrane association is mediated by a covalent conjugation to a lipid, phosphatidylethanolamine.[58] Both LC3I and LC3II are seen in nonstarved cells, but during autophagy induction the proportion of LC3II increases[28] (Fig. 7A).

Another mammalian autophagy protein, Apg5/Atg5, was also recently identified and characterised.[59] Apg5 localises to small crescent-shaped membrane structures that may be the mammalian preautophagosomal structure, the compartment that delivers membrane for newly forming autophagosomes. In developing autophagosomes, Apg5 localises mainly to the cytoplasmic side of the outer limiting membrane. Apg5 dissociates from the membranes of sealed autophagosomes. Interestingly, Apg5 is conjugated covalently to Apg12/Atg12 by a lysine located in the middle of Apg5.[60] These conjugates are then linked to a polymer by a third protein, Apg16L.[61,62] It has been proposed that this polymer acts as membrane coat, which assists membrane curvature during autophagosome formation.

The tumor suppressor gene beclin 1 is the mammalian homolog of Apg6/Atg6. Interestingly, like the yeast Apg6, beclin 1 forms a complex with the class III PI3-kinase Vps34.[63,64] Vps34 function is needed for autophagosome formation,[29,36] although the precise role of this kinase is not known.

The remainder of the known mammalian autophagy genes (Table 1) are enzymes or other factors assisting in LC3 processing or modification of LC3I to LC3II, or conjugation of Apg5 to Apg12.

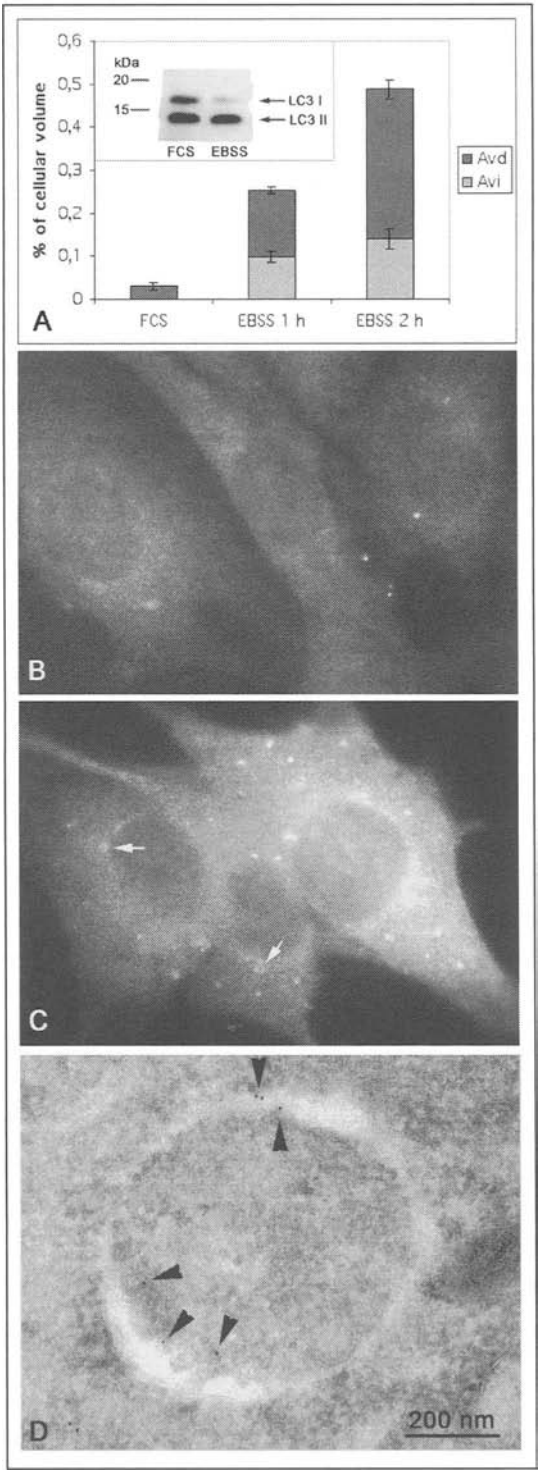

Figure 7. Detecting autophagy with LC3 antibodies in normal rat kidney (NRK) cells. A) Quantitative electron microscopy was used to estimate the volume fraction of autophagic vacuoles in nonstarved (FCS) cells, and in cells starved of serum and amino acids for 1 h (EBSS 1 h) and 2 h (EBSS 2 h). The insert shows a Western blot of LC3 from nonstarved and 2-h starved cells. The soluble LC3-I and membrane-bound LC3-II are indicated. B,C) Immunofluorescence staining of endogenous LC3 in nonstarved (B) and 2-h starved (C) cells. The arrows in panel C indicate ring-like structures, which suggest that LC3 is decorating the limiting membranes of autophagosomes. D) Immunogold labelling of LC3 indicates that the label (arrowheads) is associated with both the outer and inner limiting membrane of autophagosomes. Isei Tanida and Takashi Ueno kindly provided anti-LC3 antibodies. Stefanie Jäger carried out the experiments for panel A, insert, and panels B-C.

Table 1. Known mammalian autophagy genes

Mammalian Autophagy Gene	Yeast Gene Old / New Name	References	Function of the Gene Product
Apg3	Apg3/Aut1/Atg3	70	Autophagosome formation. Mediates LC3 modification and conjugation of Apg5 to Apg12.
Apg4/autophagins	Apg4/Aut2/Atg4	71,72	Autophagosome formation. Assists LC3 modification by cleaving the C-terminus to expose glycine.
Apg5	Apg5/Atg5	59	Autophagosome formation. Localises to isolation membranes that are forming new autophagosomes. Forms a complex with Apg12.
Beclin 1	Apg6/Vps30/ Atg6	45,47,63,64,73	Autophagy induction or autophagosome formation. Forms a complex with class III PI3-kinase Vps34. A tumor suppressor gene in mammals.
Apg7	Apg7/Atg7	74,75	Autophagosome formation. Mediates conjugation of Apg5 to Apg12, and LC3 modification.
MAP-LC3	Aut7/Apg8 Atg8	28	Autophagosome formation. Localises to the limiting membranes of autophagosomes.
Apg10	Apg10/Atg10	76,77	Autophagosome formation. Mediates conjugation of Apg5 to Apg12 and facilitates LC3 modification.
Apg12	Apg12/Atg12	78	Autophagosome formation. Localises to isolation membranes that are forming new autophagosomes. Forms a complex with Apg 5.
Apg16L	Apg16/Atg16	61	Autophagosome formation. Links together Apg5-Apg12 complexes to form polymers.

Quantitation of Autophagy

The classical ways to measure autophagy are quantitative electron microscopy and the degradation rate of long-lived proteins. Although electron microscopy is work-intensive and requires dedicated personnel and expensive equipment, it is by far the most sensitive and accurate way to detect the induction of autophagy (Fig. 7A). With most continuous cultured cell lines, the cytoplasmic volume fraction of autophagic vacuoles increases up to 10-fold during a 2-h amino acid starvation[32] (Fig. 7A). At the same time, the degradation rate of long-lived

proteins increases up to two-fold.[32] However, to find out how much of the increased proteolysis is due to autophagy, an additional sample incubated with an autophagy inhibitor (3-methyladenine or wortmannin) must be included. Autophagic protein degradation is then revealed as the difference between the amino acid starved cells without and with the inhibitor[32] (Fig. 6B). Quantitative electron microscopy estimates the accumulation of autophagic vacuoles, which can be elevated either due to increased formation of autophagosomes, or decreased degradation in late autophagic vacuoles. Degradation of long-lived protein is a measure of the degradation rate of cytoplasmic constituents, i.e., delivery of cytoplasm to lysosomal compartment or the capacity of lysosomes to degrade the delivered substrates. In other words, it is possible to have increased accumulation of autophagic vacuoles but decreased degradation of long-lived proteins in the same cells, or vice versa.

Another biochemical method to estimate autophagic activity was developed by Seglen. This assay measures the sequestration of a soluble cytoplasmic marker to a membrane-bound and thus sedimentable cell fraction.[65,66] The cytoplasmic markers used include lactate dehydrogenase (enzyme activity assay) or a radioactive sugar introduced by electroporation (radiactivity measurement). For the assay cells are usually incubated in the presence of lysosomal enzyme inhibitors such as leupeptin to prevent loss of the marker protein after delivery to the lysosomal compartment. This assay thus follows the rate of formation of new autophagosomes but does not tell anything about the degradative capacity in late autophagic vacuoles.

The fluorescent dye, monodansylcadaverine (MDC), was suggested to be a specific marker for autophagic structures.[67,68] However, several researchers have found that this dye is not specific for autophagic vacuoles. In fact the staining is punctate irrespective of whether the cells are induced for autophagy or not, although the amount of punctae may increase during starvation.[68] Thus the increase in MDC-stained punctae may indicate increased autophagy, but as such MDC is not a specific marker of autophagic vacuoles.

LC3 was introduced as the first protein that localises specifically to autophagosome membranes. A small amount of LC3 is present also in late autophagic vacuoles, because the portion of LC3II associated with the inner limiting membrane of autophagosomes is trapped inside the vacuoles.[28] Immunofluorescence staining for endogenous LC3, or LC3 Western blotting, can be used to assay autophagy (Fig. 7A-C). However, as demonstrated by Figure 7A, although the cytoplasmic volume fraction of autophagic vacuoles increases 10-fold during 2-h amino acid starvation, the amount of LC3II does not change, but instead a decrease in LC3I is observed. In other cell lines such as HeLa cells the amount of LC3II seems to better correlate with the amount of autophagic vacuoles.[28] It seems however that LC3II Western blotting is not as sensitive measure for autophagic activity as quantitative electron microscopy, and that it may be better suited for a limited number of cell lines or tissues. LC3 immunofluorescence is heavily dependent on high-quality antibodies, and it does not work in all cell types, possibly due to low expression levels of the protein. GFP-tagged LC3 expression can offer a way around these problems, and this approach has been successfully used in transgenic mice.[46] However, when transiently expressed in cultured cells, LC3-GFP forms punctate structures also without autophagy induction. This is possibly due to autophagy induction by LC3 overexpression. The number of punctae does however seem to increase during starvation, so combined with quantitation, the fluorescence pattern of transiently expressed LC3-GFP also might be useful as an autophagy assay. In any case, as a first specific autophagosome marker, LC3 is extremely useful for helping to identify and define autophagic structures in both fluorescence and electron microscopy. Various structures have been misidentified as autophagic vacuoles in the literature, mostly because of lack of specific marker proteins as well as morphological expertise to correctly interpret conventional plastic-embedded EM images.

All in all, LC3 immunofluorescence and Western blotting may offer a rapid method for screening autophagic activity, but it is recommendable to confirm the result using one or two of the established autophagy assays, quantitative electron microscopy, long-lived protein degradation, or enzyme sequestration assay.

Guidelines for Identification of Autophagic Vacuoles in Transmission Electron Microscopy

By definition, autophagic vacuoles are membrane-bound structures that *contain cytoplasm*, ribosomes, endoplasmic reticulum (ER) membranes, mitochondria, or occasionally peroxisomes. Structures that do not fulfil the latter criterion should not be classified as autophagosomes or autophagic vacuoles. In other words, vesicles containing exclusively membrane whorls, small vesicles, or undefined amorphous debris should not be called autophagic vacuoles and cannot be used as an evidence of autophagic activity. When looking at plastic-embedded specimens, it is relatively easy to identify autophagic vacuoles because ribosomes have a good contrast after staining with uranyl acetate and lead citrate. Structures surrounded by a single, double, or multiple membrane, containing clearly identifiable cytoplasmic constituents, are autophagosomes or autophagic vacuoles (Fig. 3A-D). The morphology of cytoplasmic constituents is not so clear in late autophagic vacuoles, but after careful examination it is possible to recognise partially degraded rough ER remnants (Fig. 3D, asterisks) or mitochondria. Mitochondria can be used also for identification in cryosections, where the ribosomal contrast is very low (Fig. 4C, m). Immunogold labelling of a cytoplasmic protein[6] to demonstrate the cytoplasmic contents, or of LC3 (Fig. 7D), can also be used for identification of autophagic vacuoles in cryosections.

Future of Autophagy

More than thirty years after the initial description of autophagy in mammalian cells by De Duve,[69] we finally have the first specific marker proteins and genetic tools to elucidate the mechanisms and functions of this fascinating cellular process. Many other mammalian homologs of the yeast autophagy genes are likely to be identified in the near future. This will further assist in identifying autophagic structures and in correctly estimating the autophagic activity in cell lines and tissues. The known physiological roles and functions of autophagy are thus likely to increase in number and diversity.

Acknowledgements

I thank Paul Saftig and Paul Steven Hartley for helpful comments on the manuscript, Isei Tanida and Takashi Ueno for LC3 antibodies, and Stefenie Jäger for providing material for Figure 7. The work presented in this review was supported by Jenny and Antti Wihuri Foundation and The Royal Society.

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CHAPTER 15

Chaperone-Mediated Autophagy

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Abstract

Many lysosomal and nonlysosomal pathways degrade intracellular proteins and this variety allows all cell proteins to be proteolysed at various speeds in response to different stimuli. Lysosomes, which are present in almost all eukaryotic cells, are major sites of intracellular proteolysis. They are quite heterogeneous and participate in protein catabolism by several mechanisms. By chaperone-mediated autophagy, lysosomes selectively degrade proteins with KFERQ-like sequences (about 25-30% of all cell proteins) by a mechanism which resembles the transport of proteins into mitochondria and other organelles since it requires (a) protein receptor(s), cytosolic and lysosomal chaperones and ATP-Mg^{++}. This pathway appears to be only active in certain cells under specific conditions, but its quantitative importance in protein turnover, in comparison to other lysosomal and nonlysosomal pathways, is still unknown.

Introduction

Proteins are continuously being degraded within the cells by various proteases at vastly different rates, usually expressed in terms of half-lives which range from a few minutes to many days (Table 1). Although there are hundreds of soluble and organellar proteases in a typical mammalian cell, proteasomes and lysosomal cathepsins are the most important in protein turnover. However, other proteases, including calpains, which participate in Ca^{2+}-mediated proteolysis, and the mitochondrial proteases, also degrade certain proteins.

In the last 20 years most research in intracellular protein degradation has been focused on the ubiquitin-proteasome pathway and its basic steps have been extensively reviewed.[1,2] In this pathway, proteins are targeted for proteolysis by the assembly, on surface-exposed lysines, of polyubiquitin degradation signals that are subsequently recognized by the 26S proteasome. In addition, proteasomes, which are multi-subunit proteolytic complexes with different molecular forms, degrade proteins by ubiquitin-independent mechanisms. This was first shown with ornithine decarboxylase,[3] the key enzyme in the biosynthesis of polyamines, but more recently with other proteins such as the cyclin-dependent kinase inhibitor p21[4] and the proto-oncoprotein c-Fos.[5] Therefore, there are multiple proteasomal degradative pathways.

Although lysosomes (Fig. 1), with their strong proteolytic capacity, were discovered in the early fifties of the past century by Christian de Duve and coworkers, the interest on these organelles in relationship with intracellular proteolysis declined after the discovery, thirty years later, of an ubiquitin-dependent proteolytic pathway (see above) which required ATP. The best established lysosomal proteolytic pathway involves sequestration by segregating structures of large areas of cytoplasm, typically including whole organelles.[6] This process, called (classical) autophagy or macroautophagy, has been extensively studied in the last few years, mainly due to

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Table 1. Half-lives for some rat liver proteins

Protein/s	Localization	Half-Life
Ornithine decarboxylase	Cytosol	10 min
Fructose 1,6-bisphosphatase	Cytosol	30 min
δ-aminolevulinate synthetase	Mitochondria	70 min
Tyrosine aminotransferase	Cytosol	90 min
Tryptophan oxygenase	Cytosol	120 min
Hydroxymethyl CoA reductase	Endoplasmic reticulum	150 min
Phosphoenol pyruvate carboxykinase	Mitochondria	5 h
Dihydroorotase	Cytosol	12 h
Glucose 6-phosphate dehydrogenase	Cytosol	15 h
Ornithine-oxo-acid aminotransferase	Mitochondria	19 h
Alanine aminotransferase	Mitochondria	20 h
Glutamate dehydrogenase	Mitochondria	24 h
Glucokinase	Cytosol	33 h
Acetyl-CoA carboxylase	Cytosol	48 h
Cytochrome P450	Endoplasmic reticulum	50 h
Adenosine triphosphatase (F_1)	Mitochondria	60 h
Catalase	Peroxisomes	60 h
Malate dehydrogenase	Mitochondria	62 h
Cytochrome c reductase	Endoplasmic reticulum	70 h
Arginase	Cytosol	96 h
α-glycerophosphate dehydrogenase	Mitochondria	96 h
Cytochrome b_5	Mitochondria	122 h
Cytochromes bc_1	Mitochondria	132 h
Cytochrome c oxidase	Mitochondria	134 h
Carbamyl phosphate synthetase	Mitochondria	185 h
Proteasomes	Cytosol/Nucleus	199 h
Ornithine transcarbamylase	Mitochondria	209 h
β-glucuronidase	Lysosomes	360 h
Lactate dehydrogenase (isozyme 5)	Cytosol	384 h
NAD glycohydrolase	Microsomes	432 h
Liver homogenate	——	80 h
Nuclear homogenate	——	115 h
Mitochondrial homogenate	——	90 h
Microsomal homogenate	——	45 h
Cytosolic homogenate	——	90 h

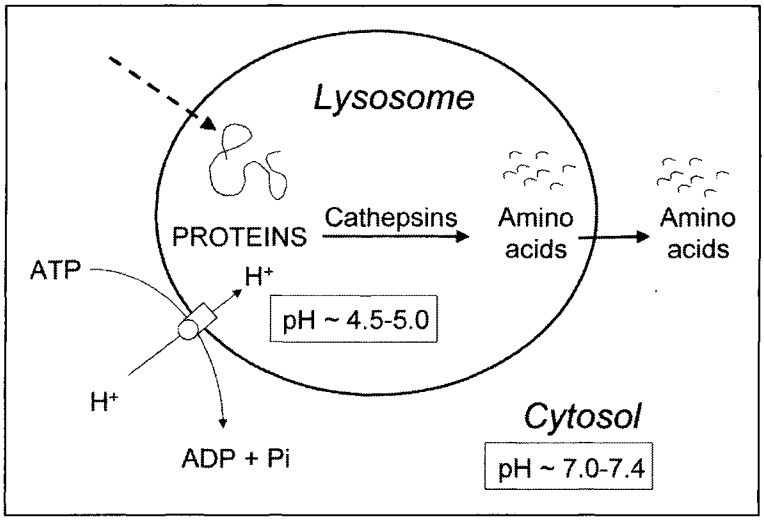

Figure 1. Lysosomal degradation of proteins. Proteins are incorporated into lysosomes by various pathways (see Fig. 2). They are degraded to amino acids in the lysosomal lumen by several lysosomal proteases (generally called cathepsins), which are more active at acid pH. The acid pH, which is maintained by an ATP-driven proton pump in the lysosomal membrane, may also affect the conformation of proteins and aid in their breakdown. Amino acids exit from the lysosome to the cytosol for further metabolism.

the availability of yeast mutants[7-9] and it is discussed in Chapter 4 of this book. Therefore, it will not be further commented here.

In addition to macroautophagy, other mechanisms (Fig. 2) have been described whereby lysosomes could degrade intracellular proteins, including heterophagy or endocytosis, crinophagy, microautophagy and chaperone-mediated autophagy.[10,11] Endocytosis and crinophagy are specific routes to degrade certain plasma membrane proteins and secretory proteins, respectively. Endocytosis is the degradative route followed by extracellular proteins, but also by certain plasma membrane proteins which, unlike the transferrin and the low density lipoprotein receptors, do not recycle back to the plasma membrane for reuse. Crinophagy degrades secretory proteins, when the demands for these proteins decline, by a process involving fusion of secretory granules with endosomes and/or lysosomes instead of with the plasma membrane. Microautophagy[12] and chaperone-mediated autophagy degrade cytosolic proteins by two different mechanisms and, compared to macroautophagy, less is known concerning both pathways. By microautophagy, portions of cytoplasm, sometimes including peroxisomes (a process also called pexophagy[13]), are directly internalysed into the lysosomal lumen by various modifications of the lysosomal membrane, producing intralysosomal vesicles which are degraded. Chaperone-mediated autophagy is the subject of this chapter and will be discussed below.

Evidence for the participation of lysosomal pathways different from macroautophagy in intracellular proteolysis are provided by differences in the inhibitions obtained with agents which affect all lysosomes (e.g., NH_4Cl and leupeptin) and those which something more specifically inhibit macroautophagy (3-methyladenine). Based on these data, and depending on the growth conditions of the cells, it could be calculated in human fibroblasts that lysosomal pathways different from macroautophagy degrade long-lived proteins (pulse-labelled for 48 h and chased for 24 h to eliminate short-lived proteins) in a range from about 5-10% (in exponentially growing cells deprived of amino acids and serum for 4 h) to 35% (in confluent cells deprived of serum but not of amino acids for 24 h).[14] In addition, work with Atg5-deficient mouse embryonic stem cells, which are completely blocked in macroautophagy, has shown

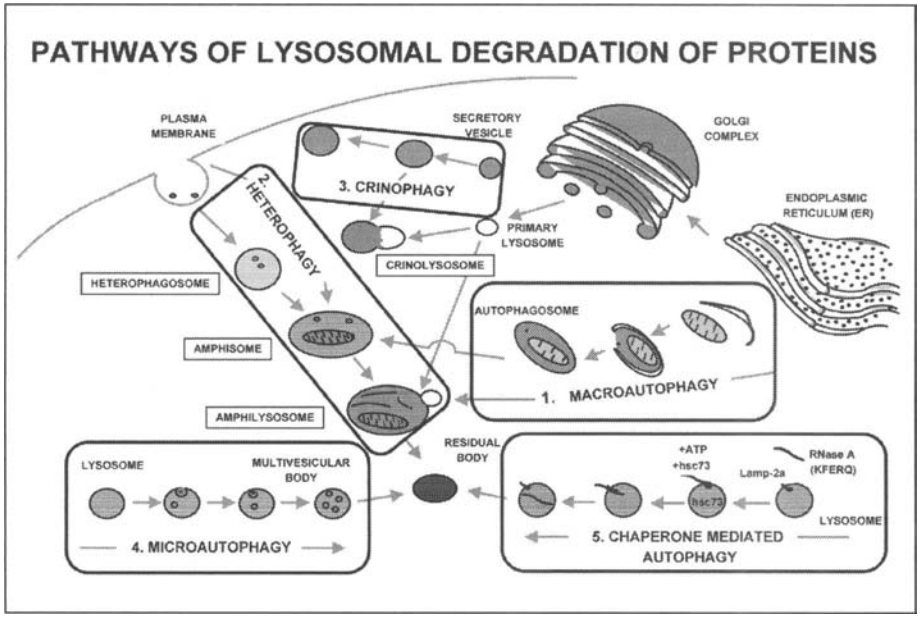

Figure 2. Lysosomes take up intracellular proteins by various pathways: macroautophagy, heterophagy, crinophagy, microautophagy and chaperone-mediated autophagy. See text for details.

that lysosomal pathways different from macroautophagy account for 30-40% of starvation-induced lysosomal protein degradation.[15]

In this review, we will concentrate on one of those lysosomal pathways different from macroautophagy, which was formerly called selective pathway for lysosomal proteolysis and lysosomal pathway mediated by the 73-kDa heat shock cognate protein. Since 2000 it has been called chaperone-mediated autophagy. The reader is also referred to excellent earlier reviews on this subject.[16-18]

Distinctive Characteristics of Chaperone-Mediated Autophagy

In this pathway, and contrary to other lysosomal proteolytic pathways, the proteins to be degraded are not surrounded by a segregating structure which either fuses with (macroautophagy, heterophagy, crinophagy) or is degraded within (microautophagy) endosomes/lysosomes. Here, these proteins directly traverse the lysosomal membrane from the cytosol into the lysosomal lumen. The rationale for such a pathway relies on the fact that proteins can either cosynthetically or post-synthetically cross many cellular membranes (e.g., mitochondria, chloroplasts, peroxisomes, endoplasmic reticulum) under appropriate conditions to reach their proper intracellular location (Fig. 3). It is well established, however, that resident proteins are transported to lysosomes *via* vesicles. Even two lysosomal proteins, aminopeptidase I and α-mannosidase which in yeast are synthesized in the cytosol, do not cross the vacuolar membrane but are instead transported to the vacuole *via* autophagosomes by the cytoplasm to vacuole targeting (Cvt) pathway.[19,20] However, it is possible that certain cytosolic proteins directly traverse lysosomal membranes for their degradation and the experimental evidences are presented in the following paragraphs.

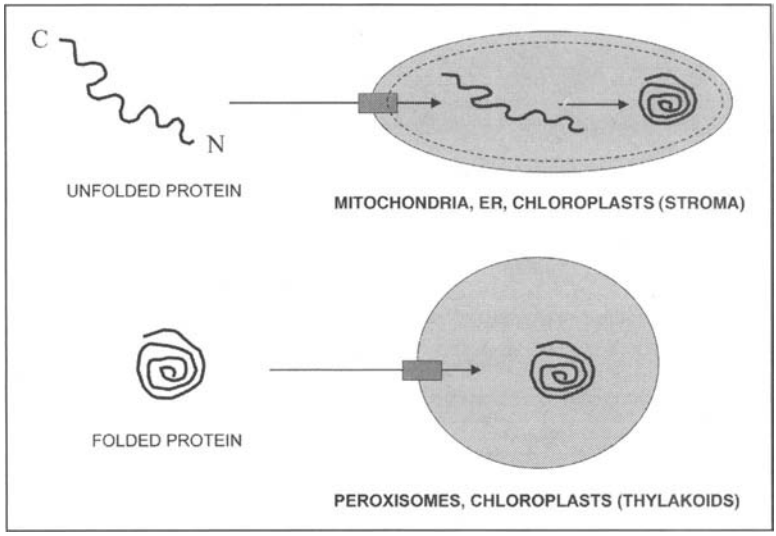

Figure 3. Resident proteins which have to enter into cytosolic organelles require the movement of the protein across the organelle membrane/s using a transport machinery. Transport of proteins into mitochondria, chloroplasts or endoplasmic reticulum (ER) requires prior unfolding of the protein. However, proteins in a folded conformation are transported into peroxisomes or through the thylakoid membranes of the chloroplast.

Lysosomes Can Take Up Selectively Ribonuclease A and Other Proteins from Cytosol

Macroautophagy, the major and most evident lysosomal proteolytic pathway is mainly a bulk degradation process. Therefore, and in contrast to the degradation by proteasomes, the lysosomal degradation of proteins has been usually, but erroneously, considered to be exclusively non selective. In mammalian cultured cells, serum withdrawal increases protein degradation mainly because activation of lysosomal degradation. Evidence for a selective transport of cytosolic proteins into lysosomes in mammalian cells was obtained when ribonuclease A (RNase A) and other proteins were microinjected into confluent human diploid fibroblasts (IMR-90 cells) and their degradation was analysed. In these experiments, some proteins (RNase A, cytosolic aspartate aminotransferase) were degraded in the absence of serum at increased rates while others (bovine serum albumin, lysozyme, ovalbumin, insulin A chain) were not.[21] In other words, there was selectivity in the degradation of certain substrates. Using various approaches it was concluded that RNase A was primarily degraded by lysosomes.[22] These studies included analysis of the acid-soluble radioactivity accumulated into isolated subcellular fractions after microinjection of RNase A tagged with the [^{3}H]-radiolabeled inert trisaccharide raffinose (which is impermeable through lysosomal membranes) and also inhibition of lysosomes by increasing their pH with ammonium chloride. Therefore, it appeared that lysosomes could selectively internalise certain cytosolic proteins.

An Amino Acid Sequence of Ribonuclease A Is Essential for Its Selective Lysosomal Targeting

RNase A (Fig. 4) is cleaved by subtilisin between residues 20 and 21 to produce RNase S-peptide (residues 1-20) and RNase S-protein (residues 21-124). The half-lives of RNase S-peptide and RNase S-protein microinjected into confluent fibroblasts were, respectively, 62 and 89 h in the presence of serum and 30 and 90 h in the absence of serum.[23] Thus, like RNase

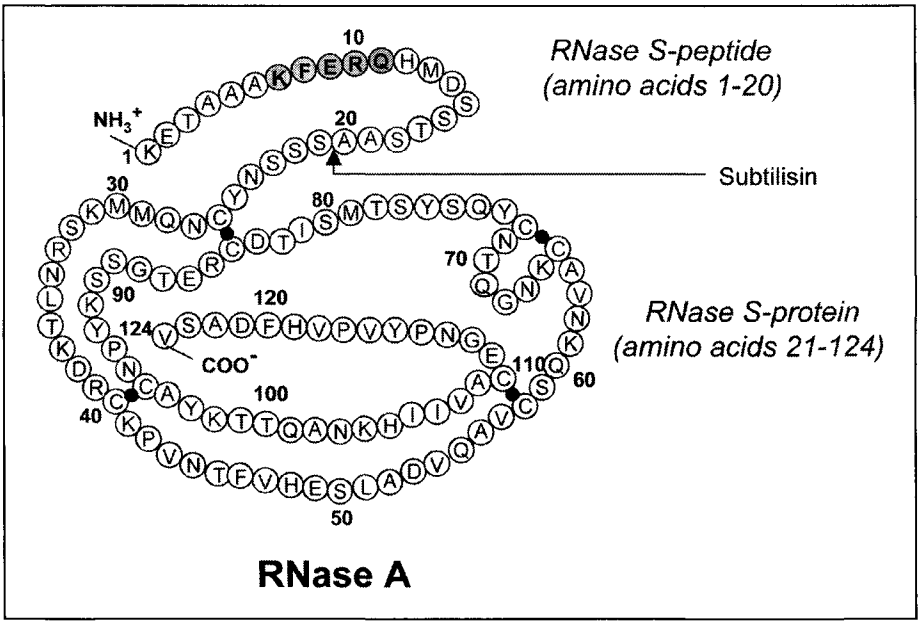

Figure 4. Structure of RNase A. The 124 amino acid residues and the localization of the disulfide bridges and the KFERQ sequence are indicated. The serin-protease subtilisin (EC 3.4.21.14) cleaves RNase A between residues 20 and 21 (arrow) producing RNase S-peptide (residues 1-20), which contains the KFERQ sequence, and RNase S-protein (residues 21-124), which does not contain it.

A, the RNase S-peptide, but not the RNase S-protein, was degraded at increased rates in the absence of serum. Moreover, when the 20 amino acids of the RNase S-peptide were covalently conjugated to other polypeptides, it was found that they increased the degradation of these proteins under serum withdrawal.[24] Experiments with synthetic peptides at increasing concentrations identified the pentapeptide KFERQ as the essential region, since it inhibited the enhanced degradation of RNase A in the absence of serum.[25] Therefore, it was concluded that, under serum deprivation in confluent fibroblasts, degradation of RNase A occurs by a specific and saturable lysosomal pathway which recognizes its KFERQ sequence. This exact sequence is only found in the pancreatic RNase A family. However, other proteins which are also degraded more rapidly in response to serum withdrawal contain a sequence which could be related to KFERQ.[26,27] The actual KFERQ-like sequences include Gln (or Asn, in some sequences but not in others) at the end or at the beginning of the sequence and, in any order, an acidic (Glu or Asp), a basic (Lys or Arg), an hydrophobic (Val, Ile, Leu or Phe) and either a second hydrophobic or a second basic amino acid.[18] Although, based on X-ray analysis, it has been postulated that these sequences are unlikely to be exposed to the degradation machinery because one or more residues are buried in the protein structure,[28] it can be argued that for a functional protein to be degraded in a regulated fashion additional covalent or noncovalent modifications are necessary which may expose these residues.

Which are the substrates of the selective lysosomal degradation pathway? It has been found that about 25-30% of cytosolic proteins contain a sequence motif related to the pentapeptide KFERQ since they were immunoprecipitated with antibodies towards the KFERQ sequence.[26] About fifteen of these substrates have been identified and include glycolytic enzymes, transcription factors and their regulatory proteins, signalling molecules, proteases, etc. Striking evidence for the importance of the KFERQ-like sequences in chaperone-mediated autophagy

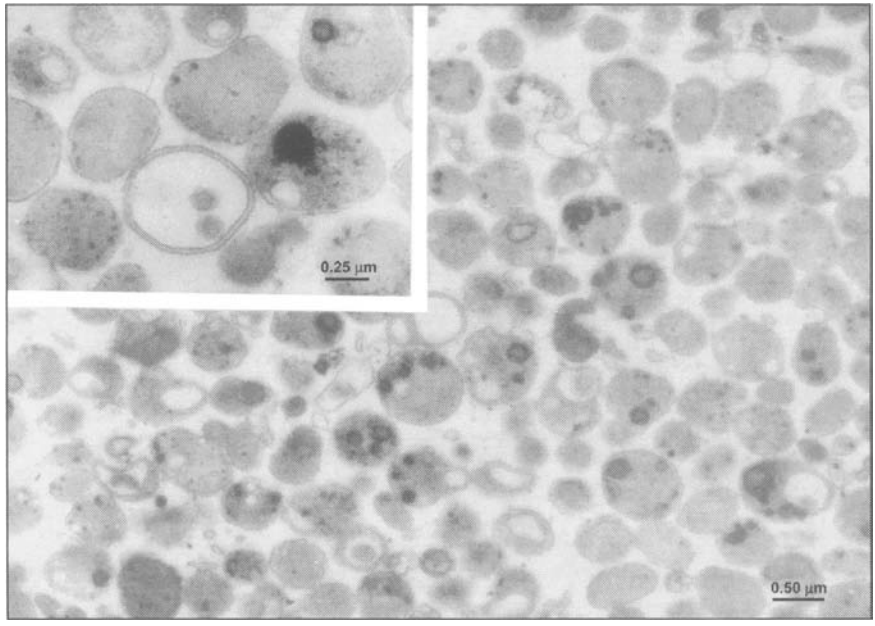

Figure 5. Electron microscopic appearance of isolated lysosomes from rat liver obtained by centrifugation of a mitochondrial-lysosomal fraction through a discontinuous metrizamide gradient. The preparation consists almost entirely of lysosomes with different morphology, but mostly dense bodies. The degree of purification of lysosomes, relative to the original rat liver homogenate, is about 53 fold, based on measurements of marker enzymes, and the yield is about 7%. The inset shows the isolated lysosomes at higher magnification.

was provided by the family of annexins: only annexins containing KFERQ-like sequences (annexins II and VI) are degraded by chaperone-mediated autophagy, while those without such sequences (annexins V and XI) are not.[29]

The Selective Lysosomal Pathway for the Degradation of Proteins Containing KFERQ-Like Sequences Is Active in Various Cell Types under Certain Conditions

The selective lysosomal pathway was found to be active in some mammalian confluent cells in culture during serum deprivation but not in others. Thus, it is active in CHO cells and skin fibroblasts but to a lesser degree than in lung fibroblasts, while COS cells have no detectable activity.[30] Also, and although the pathway was found to be active in some rat tissues (liver, heart) subjected to starvation for more than 24 h (prolonged starvation), it was not in skeletal muscle.[31,32] In addition, conditions have been described where the selective lysosomal pathway is inhibited. Thus, in cultured renal epithelial cells, the epidermal growth factor suppresses this pathway[33] and it has been also reported that the pathway decreases in confluent cultures of senescent human fibroblasts[34] and in rat liver with age.[35]

Molecular Chaperones Participate in the Selective Lysosomal Transport of Proteins

The transport of proteins from cytosol into lysosomes has been also reconstituted with isolated lysosomes from human fibroblasts and rat liver (Fig. 5). It requires molecular chaperones and thus resembles protein import for residence in organelles such as mitochondria,

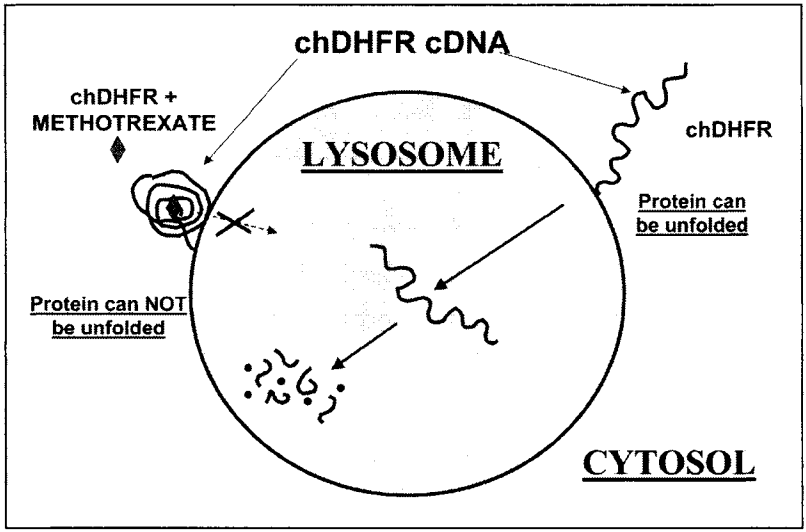

Figure 6. An in vitro synthesized chimeric protein (chDHFR) of dihydrofolate reductase (DHFR) is transported into isolated rat liver lysosomes by chaperone-mediated autophagy (right side). The DHFR substrate analogue methotrexate complexes with high affinity with the enzyme and locks it in a folded conformation (left side). Without methothrexate the chimeric protein can be unfolded and is transported through the lysosomal membrane. In the presence of methotrexate, however, the protein remains in a folded conformation which still binds to the lysosomal membrane, but the transport through the lysosomal membrane is strongly reduced. It appears, therefore, that the lysosomal uptake of this protein by chaperone-mediated autophagy mainly occurs by passage of the unfolded protein through the lysosomal membrane.

endoplasmic reticulum and chloroplasts. Like in these pathways, a sequence (KFERQ-like motifs) is recognized by cytosolic chaperones (the constitutively expressed molecular chaperone of 73-kDa hsc73).[36] Hsc73 interacts with RNase A, RNase S-peptide, aspartate aminotransferase and pyruvate kinase, while no binding was observed with polypeptides which lack a KFERQ-like region, such as ovalbumin and lysozyme. In contrast, other hsp70 family members, such as hsp70, grp78 and dnaK, among others, did not bind to RNase S-peptide.[37]

In addition, the degradation of protein substrates of the pathway by lysosomes requires, for still unknown reasons, ATP/Mg^{++}.[36] It is possible that the interactions of hsc73 and protein substrates are mediated by cycles of ATP binding and hydrolysis and/or that ATP may be required to maintain active the lysosomal ATP-dependent proton pump.

Protein substrates must be unfolded to be translocated across the lysosomal membrane,[38] but this is not required for their binding to the lysosomal membrane (Fig. 6). Therefore, unfolding of the protein may occur at the lysosomal surface. In this regard, a multi-molecular chaperone complex at the cytosolic side of the lysosomal membrane is required for translocation of substrate proteins.[39] This complex includes hsc73, hsp90, hsp40, the hsp70-hsp90 organizing protein (Hop), the hsc70 interacting protein (Hip) and the Bcl2-associated athanogene 1 protein (BAG-1) and antibodies against Hip, Hop, Hsp40 and hsc73 blocked the transport of protein substrates into isolated lysosomes.[39]

Once bound to the lysosomal membrane, the substrate protein is transported into lysosomes assisted by another chaperone, the lysosomal hsc73 (ly-hsc73), which is required for the complete lysosomal transport of substrate proteins.[40,41] If ly-hsc73 is absent or experimentally blocked by endocytosis of specific antibodies, no transport of substrates takes place.[40] The levels of ly-hsc73 increase after nutritional deprivation and contribute to the augmented lysosomal degradation rates under those conditions. By analogy with the biosynthetic transport of

proteins to mitochondria, ly-hsc73 could interact with the emerging protein, providing the driving force to pull the transport intermediate into lysosomes, preventing its back movement and leading the process irreversible. However, this would probably require the availability of ATP to be hydrolysed within lysosomes, something which is difficult to imagine in the lysosomal degradative milieu. In any case, the role of molecular chaperones in the transport of proteins to lysosomes justifies the name of chaperone-mediated autophagy for the pathway.

LAMP2a Is a Receptor Protein in the Lysosomal Membrane for the Entry of Proteins from the Cytosol into Lysosomes by Chaperone-Mediated Autophagy

RNase S-peptide specifically binds to the lysosomal membrane and blocks the binding and uptake of other protein substrates.[42,43] Binding assays with lysosomal membranes and two substrates of the pathway (glyceraldehyde-3-phosphate dehydrogenase and RNase A) showed that the chaperone-substrate protein complex binds to the lysosomal membrane protein LAMP2.[30] No binding was observed to the related lysosomal membrane protein LAMP1.

LAMP2 belongs to the group of lysosomal-associated membrane proteins (LAMPs) with very similar structural characteristics: they are type I integral membrane proteins, highly glycosylated (in LAMP2, the protein core accounts for only 40 kDa of the mature glycosylated protein of 96 kDa) and are composed of a large lumenal domain, a single transmembrane region of about 20 amino acids and a short carboxyl terminus tail at the cytosolic side of the lysosomal membrane of about 10-12 amino acids.[44-46] The *LAMP2* gene undergoes alternative splicing resulting in three different mRNAs, encoding different isoforms of LAMP2 (a, b and c) with high amino acid sequence identity in their lumenal region but different transmembrane and cytosolic regions,[47,48] which are expressed at different levels in different tissues.[48,49] The level of the receptor at the lysosomal membrane is the rate limiting step in the binding and the uptake of the substrate proteins and the levels of LAMP2a (but not of other LAMP2 isoforms) in the lysosomal membrane directly correlate with rates of chaperone-mediated autophagy in a variety of conditions.[50,51] This is regulated by changes in the lysosomal degradation of LAMP2a and in its dynamic distribution between the lysosomal membrane and matrix,[50] where a portion of LAMP2a is located.[52] Therefore, since a translocon has not yet been identified in chaperone-mediated autophagy and LAMP2a has been found to homomultimerize, it could be possible that this protein has a dual role as receptor and translocon in this proteolytic pathway.

Protective protein/cathepsin A associates on the lysosomal membrane with LAMP2a and triggers its degradation by cleaving LAMP2a near the luminal and transmembrane domain boundaries.[53] This cleavage releases a truncated LAMP2a into the lysosomal matrix where it is degraded by lysosomal proteases. In addition, cathepsin A is required to initiate another cleavage on the C-terminal region of LAMP2a in cooperation with an unidentified metalloprotease at the lysosomal membrane.[53]

Specific antibodies against the short cytosolic tail of LAMP2a or competition with a synthetic peptide of the same amino acid sequence as the tail, results in blocking of substrate uptake and degradation in lysosomes.[30] Four positively-charged amino acids (KRHH) uniquely present in the cytosolic tail of LAMP2a are required for the binding of substrate proteins. RNase S-peptide does not bind to LAMP2[30] and, therefore, other components of the protein binding and import machinery may exist.

Mice deficient in LAMP2 were generated to investigate the functions of this protein[54] and have revealed roles for LAMP2 in macroautophagy and lysosomal biogenesis.[46,54,55] Some of the observations with these mice can be attributed to specific LAMP2 isoforms. Thus, certain mutations in a *LAMP2b* specific exon, cause Danon's disease, a myopathy in which autophagic vacuoles accumulate in skeletal and cardiac muscle.[56] However, experiments carried out in our laboratory in collaboration with the laboratory of Paul Saftig (Kiel University, Germany) using LAMP2-deficient and LAMP1- and LAMP2-double deficient mouse embryo fibroblasts

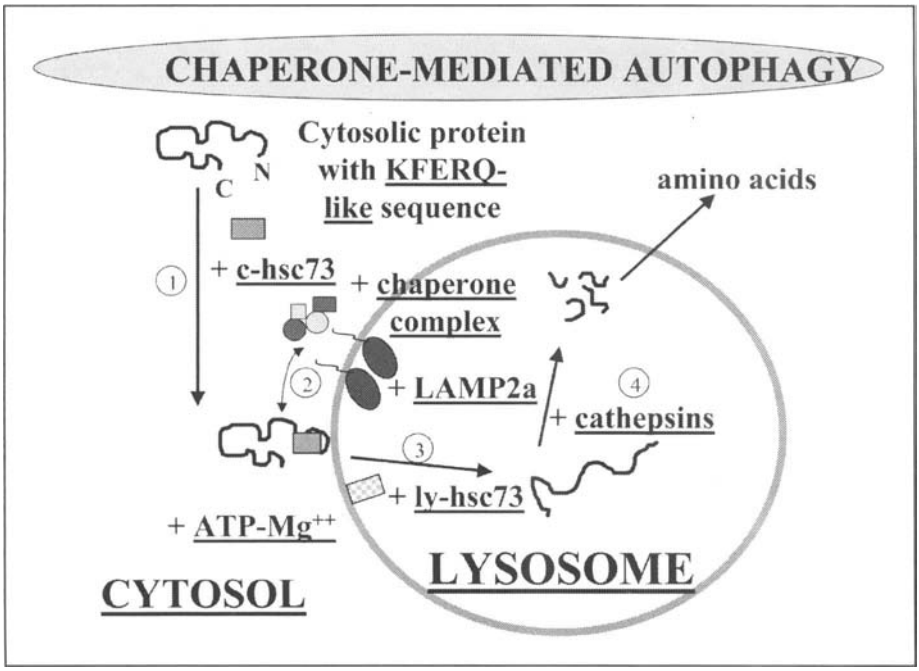

Figure 7. A possible model for chaperone-mediated autophagy. The protein interacts with the heat shock cognate protein of 73 kDa (c-hsc73)(①) and is recognised and bound to the cytosolic side of the lysosomal membrane through a complex which includes LAMP2a, probably in a homomultimeric form, and various chaperones (②). Next, the unfolded protein enters into the lysosomal matrix through the lysosomal membrane, in a still unknown way, assisted by a lysosomal form of hsc73 (ly-hsc73) (③) and is degraded by lysosomal cathepsins (④).

do not show any change, as compared to control cells or cells deficient in LAMP1, in the protein degradation rates by lysosomes under six different conditions (exponentially growing and confluent cells, 4 h or 28h serum withdrawal, 4h serum and amino acid deprivation). These results[57] indicate that in these cells, LAMP2 deficiency does not alter lysosomal protein degradation even in confluent cells under prolonged serum starvation, a condition where chaperone-mediated autophagy is activated. Alternatively, it is possible that LAMP2 is not the only receptor in chaperone-mediated autophagy or that in mouse embryo fibroblasts, like in other cells, the pathway is not active, although its proteolytic behaviour under the various conditions resembles that of human fibroblasts, where the pathway is active. Another possibility is that the deficiency in the activity of the chaperone-mediated autophagic pathway in LAMP2 (-/-) mouse embryo fibroblasts is compensated by other proteolytic pathways. Although our experiments with various inhibitors seem to exclude this with macroautophagy, proteasomes and nonlysosomal proteolytic pathways different from proteasomes, the possibility still exists that a lysosomal pathway which is different from macroautophagy, is activated to compensate for the deficiency in chaperone-mediated autophagy.

Conclusions

The lysosomal degradative pathway called chaperone-mediated autophagy (Fig. 7) appears to be unique when compared to other lysosomal pathways for the degradation of cellular proteins in that proteins to be degraded move across the lysosomal membrane. This transport

occurs by a mechanism which resembles in some respects the import of proteins synthesized on cytosolic ribosomes into mitochondria and endoplasmic reticulum, since it requires a signal sequence in the substrate proteins, a protein receptor in the lysosomal membrane, unfolding of the protein, molecular chaperones, both in the cytosol and in lysosomes, and ATP. This pathway appears to operate only in certain cell types and tissues, but not in others, especially under extreme conditions of prolonged starvation. The major question which remains to be solved is the precise quantification of the real importance of chaperone-mediated autophagy under physiological and pathological conditions in whole cells. This will require to clearly distinguish it from other lysosomal pathways. The use of LAMP2 deficient cells will be quite useful in this research.

Acknowledgements

Work in E. Knecht's laboratory is supported by the Spanish Ministerio de Ciencia y Tecnología (grants BMC2001-0816 and SAF2002-00206) and the Fondo de Investigaciones Sanitarias del Instituto de Salud Carlos III (Red de Grupos RGDM G03/212).

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