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REVIEW

Lysosomal metabolism of glycoproteins

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The lysosomal catabolism of glycoproteins is part of the normal turnover of cellular constituents and the cellular homeostasis of glycosylation. Glycoproteins are delivered to lysosomes for catabolism either by endocytosis from outside the cell or by autophagy within the cell. Once inside the lysosome, glycoproteins are broken down by a combination of proteases and glycosidases, with the characteristic properties of soluble lysosomal hydrolases. The proteases consist of a mixture of endopeptidases and exopeptidases, which act in concert to produce a mixture of amino acids and dipeptides, which are transported across the lysosomal membrane into the cytosol by a combination of diffusion and carrier-mediated transport. Although the glycans of all mature glycoproteins are probably degraded in lysosomes, the breakdown of N-linked glycans has been studied most intensively. The catabolic pathways for high-mannose, hybrid, and complex glycans have been established. They are bidirectional with concurrent sequential removal of monosaccharides from the nonreducing end by exoglycosidases and proteolysis and digestion of the carbohydrate–polypeptide linkage at the reducing end. The process is initiated by the removal of any core and peripheral fucose, which is a prerequisite for the action of the peptide N-glycanase aspartylglucosaminidase, which hydrolyzes the glycan–peptide bond. This enzyme also requires free alpha carboxyl and amino groups on the asparagine residue, implying extensive prior proteolysis. The catabolism of O-linked glycans has not been studied so intensively, but many lysosomal glycosidases appear to act on the same linkages whether they are in N- or O-linked glycans, glycosaminoglycans, or glycolipids. The monosaccharides liberated during the breakdown of N- and O-linked glycans are transported across the lysosomal membrane into the cytosol by a combination of diffusion and carrier-mediated transport. Defects in these pathways lead to lysosomal storage diseases. The structures of some of the oligosaccharides that accumulate in these diseases are not digestion intermediates in the lysosomal catabolic pathways but correspond to intermediates in the biosynthetic pathway for N-linked glycans, suggesting another route of delivery of glycans to the lysosome. Incorrectly folded or glycosylated proteins that are rejected by the quality control mechanism are broken down in the ER and cytoplasm and the end product of the cytosolic degradation of N-glycans is delivered to the lysosomes. This route is enhanced in cells actively secreting glycoproteins or producing increased amounts of aberrant glycoproteins. Thus interaction between the lysosome and proteasome is important for the regulation of the biosynthesis and distribution of N-linked glycoproteins. Another example of the extralysosomal function of lysosomal enzymes is the release of lysosomal proteases into the cytosol to initiate the lysosomal pathway of apoptosis.

Key words: catabolism/cathepsins/glycoproteins/glycosidases/lysosomal

Introduction

About 1% of the human genome is devoted to maintaining and manipulating the glycosylation of cells (Varki and Marth, 1995). The lysosomal catabolism of glycoproteins is an important part of the cellular homeostasis of glycosylation (Figure 1) (Cuervo and Dice, 1998; Mason, 1996; Winchester, 1996) as is shown by the morbidity and mortality associated with the glycoproteinoses, the diseases that result from defects in the lysosomal catabolism of glycoproteins (Cantz and Ulrich-Bott, 1990; Hancock and Dawson, 1989; Michalski and Klein, 1999; Thomas, 2001; Winchester, 2004). The lysosomal system is not the only cellular machinery for the breakdown of proteins. Protein degradation also occurs in other organelles, where it is usually restricted to organelle-specific proteins, for example, in the mitochondrion (Desautels and Goldberg, 1982; Korbel *et al*., 2004). In the cytosol and nucleus, normal short-lived proteins and abnormal proteins are conjugated to ubiquitin and delivered to the proteasome for degradation (Hilt and Wolf, 2004). The proteasome is also involved in the catabolism of incorrectly folded glycoproteins, which are transported to the cytosol if they are rejected by the quality control mechanism in the endoplasmic reticulum (ER). It is becoming clear that the lysosome and the proteasome work in conjunction in the breakdown of these incorrectly folded glycoproteins (Hirsch *et al*., 2003; Suzuki and Lennarz, 2003), illustrating the interdependence of the functions of different subcellular compartments.

Over 40 different glycosidic bonds between sugars and amino acids, involving 8 amino acids and 13 different monosaccharides, have been identified (Spiro, 2002). After the formation of the glycosidic bond, glycans or oligosaccharide chains can be built up on the sugar by the stepwise addition of monosaccharides from activated donors except for asparagine-linked glycans and glycosylphosphatidylinositol (GPI) anchors, which are transferred *en bloc* to the protein. The majority of mammalian glycoproteins contain glycans linked either by the N-glycosidic amide N of

asparagines or O-glycosidically linked to the OH-group of serine/threonine or hydroxylysine (Figure 2). All cellular glycoproteins are being turned over continuously, albeit at different rates. As far as is known, all mature, natural glycoproteins can be catabolyzed to their constituent amino acids (plus some dipeptides) and monosaccharides in the lysosomes by the action of a cocktail of enzymes, predominantly acidic hydrolases. The digestion products pass through the lysosomal membrane for reuse by the cell. In this review the transport of glycoproteins to lysosomes, the proteolysis of the polypeptide moiety, the release and breakdown of the glycans, the transport of the breakdown products out of the lysosome, and the relationship between lysosomal and cytosolic/ER degradation will be discussed.

Transport of glycoproteins to lysosomes

The location and function of a glycoprotein determine its rate of turnover and route of delivery to the lysosome. Historically, *autophagy* was the term for the nonselective process of delivering material from the cytoplasm (i.e., excluding the nucleus and plasma membrane) of eukaryotic cells to the lysosome for digestion and *heterophagy* the term for the uptake and lysosomal digestion of exogenous material. The advances in elucidating the molecular basis of membrane trafficking and organelle biogenesis and the widespread involvement of ubiquitination have blurred these definitions (Schekman, 2004) but it is still helpful from a functional standpoint to consider the catabolic fate of endogenous and exogenous glycoproteins separately.

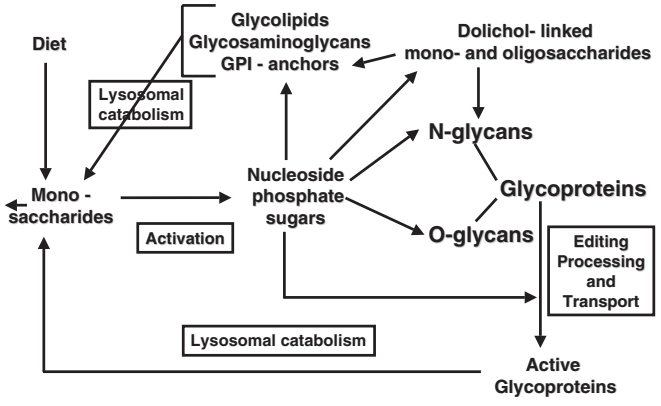

Fig. 1. The role of lysosomal catabolism of glycoproteins in the cellular homeostasis of glycosylation.

Several forms of autophagy are recognized (Cuervo, 2004; Dunn, 1994; Levine and Klionsky, 2004). In macroautophagy fractions of the cytoplasm are sequestered in newly formed vacuoles (autophagosomes) that subsequently fuse with lysosomes. Macroautophagy is an important component of cellular homeostasis and accounts for the turnover of many long-lived cellular glycoproteins in organelles. Crinophagy is a process used to regulate the rate of secretion of secretory proteins by diverting secretory granules to fuse with lysosomes/endosomes rather than the plasma membrane. Cytosolic material may also be transferred into the lysosome directly by invagination of the lysosomal membrane to form vesicles in the lumen of the lysosome (microautophagy). The vesicular membrane, which is rich in glycoproteins, is broken down to expose the cytoplasmic material, which may be a random sample of soluble material or selected whole organelles, such as peroxisomes. Microautophagy is a slow, constitutive, energy-dependent process that is essentially unselective for cytosolic proteins, including glycoproteins. In contrast, chaperone-mediated autophagy is highly selective (Cuervo and Dice, 1998). Unfolded (Salvador *et al*., 2000), soluble cytosolic proteins that possess the targeting signal, KFERQ or KFERQ-like motif, can bind to a cytosolic chaperone, the heat-shock protein, hsc73. The resultant complex binds to the short cytosolic tail of integral lysosomal membrane proteins, including the lysosome-associated membrane protein-2a (lamp-2a). The cytosolic protein is then translocated across the lysosomal membrane with the assistance of an intralysosomal form of the chaperone, hsc73, and degraded in the

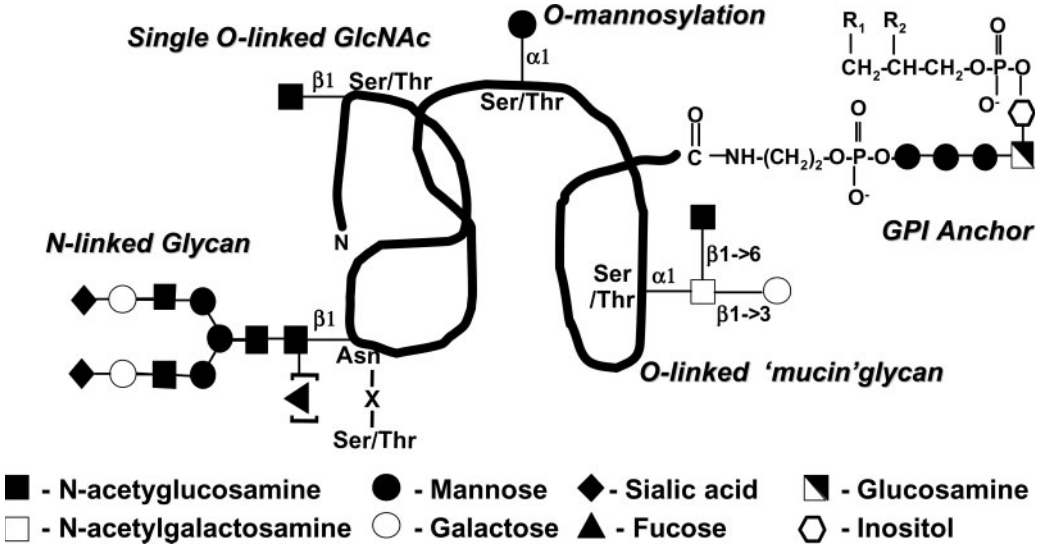

Fig. 2. The main forms of attachment of carbohydrate to protein in mammalian cells.

lysosome. The serine protease, cathepsin A, which also has a protective role toward certain lysosomal hydrolases, initiates the degradation of the receptor, lamp-2a, thereby controlling the rate of chaperone-mediated autophagy (Cuervo *et al*., 2003). About 30% of cytosolic proteins can be degraded by chaperone-mediated autophagy, and it plays an important part in the regulation of protein degradation under different physiological conditions. Fragments of glycans derived from the breakdown of incorrectly folded or glycosylated glycoproteins in the ER and cytosol are also delivered to the lysosome by a receptor-mediated mechanism for digestion (Saint-Pol *et al*., 1999) (see later discussion).

Heterophagy or endocytosis is the mechanism for delivering extracellular and plasma membrane proteins to the lysosome for catabolism (Mellman, 1996; Smythe, 1996). It encompasses phagocytosis of cellular debris and pathogenic microorganisms, receptor-mediated endocytosis via clathrin-coated pits of specific protein ligands, nonspecific fluid phase pinocytosis and macropinocytosis (Swainson and Watts, 1995) of extracellular proteins, and endocytosis by caveolae. Pinocytosis is constitutive in most cell types, whereas phagocytosis is predominantly carried out by professional scavengers in response to stimulation. All of these processes result in the formation of early endosomes (or phagosomes) where sorting of the material for delivery to the lysosome or recycling to the plasma membrane occurs (Luzio *et al*., 2001; Maxfield and Mukherjee, 2004). The endosomes mature into late endosomes, which contain the material to be digested and fuse with lysosomes to form hybrid organelles, in which digestion commences (Luzio *et al*., 2001). Subsequently lysosomes are reformed by condensation of the contents and removal of some membrane proteins by vesicular traffic.

Lysosomal catabolism of glycoproteins

Once inside lysosomes, the catabolism of glycoproteins is catalyzed by hydrolases with characteristic acidic pH optima. The low pH inside lysosomes not only provides the optimal conditions for the enzymes but also probably partially denatures the glycoproteins. The polypeptide chain is broken down by proteases and the glycan moiety by glycosidases. Any other posttranslational modifications, for example, sulfation, phosphorylation, or esterification, are removed by appropriate hydrolases. Very little is known about the precise pattern of breakdown of the polypeptide portion of any glycoprotein in vivo because there is an excess of protease activity and many of the enzymes have overlapping specificities. This probably accounts for the apparent absence of lysosomal storage diseases due directly to a generalized defect in lysosomal proteolysis. This is supported by the absence of nonspecific lysosomal protein storage in mice knocked out for a specific protease (Saftig *et al*., 1995), However, these models also indicate that some proteases have specific functions in addition to contributing to the general proteolytic capacity of cells (Turk *et al*., 2001). Often this function is restricted to certain cell types or is extracellular, explaining the lack of a general defect in protein turnover due to their deficiency. To date only four human diseases have been shown to be due directly to defects in genes encoding lysosomal proteases (Table I). In contrast, highly specifc glycosidases catalyze the hydrolysis of the different glycosidic linkages in N- and O-linked glycans in highly ordered pathways. Consequently a defect in one enzyme will block the pathway and lead to the accumulation of its substrate, resulting in a lysosomal storage disease (Winchester, 1996) (Table I and Figure 3).

Enzymology of lysosomal proteolysis

The proteolytic capacity of lysosomes comprises a mixture of endo- and exo-peptidases, called cathepsins, which act in concert to degrade proteins to a mixture of amino acids and dipeptides (Barrett *et al*., 1998; Mason, 1996) (Table II). Some cathepsins, for example, G and E, also function outside the lysosome. All of the proteases are active at an

Table I. Lysosomal storage diseases with defects in proteases or catabolism of glycoproteins

Disorder	Enzymic defect
Fucosidosis	α-L-fucosidase (E.C. 3.2.1.51)
Sialidosis (mucolipidosis I)	N-acetyl-α-neuraminidase (E.C. 3.2.1.18) (sialidase)
G_{M1}-gangliosidosis	β-D-galactosidase (E.C. 3.2.1.23)
Sandhoff disease	β-D-hexosaminidase A and B (E.C. 3.2.1.30)
α-Mannosidosis	α-D-mannosidase (E.C. 3.2.1.24)
β-Mannosidosis	β-D-mannosidase (E.C. 3.2.1.26)
Aspartylglucosaminuria	*N*-(β-*N*-acetylglucosaminyl)-1-asparaginase or aspartylglucosaminidase (E.C. 3.5.1.26)
Schindler (Kawasaki) disease	α-*N*-acetylgalactosaminidase (α-D-galactosidase B) (E.C. 3.2.1.49)
I-cell disease and pseudo-Hurler polydystrophy (mucolipidosis II and III)	UDP-*N*-acetylglucosamine-1-phosphotransferase leading to multiple enzyme deficiencies
Galactosialidosis	Protective protein/cathepsin A (E.C. 3.4.16.1)
Papillon-Lefevre syndrome	Cathepsin C (E.C. 3.4.14.1)
Ovine and murine ceroid lipofuscinosis	Cathepsin D (E.C. 3.4.23.5)
Pycnodysostosis	Cathepsin K
Infantile neuronal ceroid lipofuscinosis	Palmitoyl protein thioesterase
Late infantile neuronal ceroid lipofuscinosis	Tripeptidyl peptidase 1

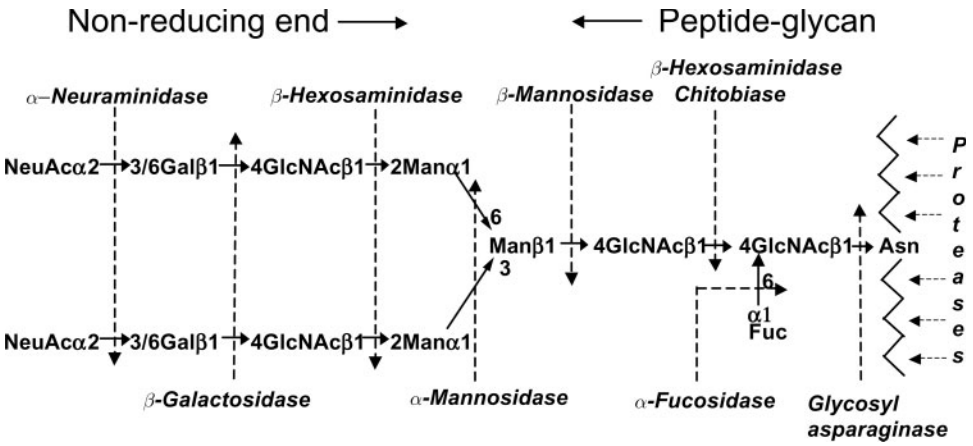

Fig. 3. Bidirectional breakdown of representative complex N-linked glycan. A specific lysososmal storage disease is associated with genetic defects in each of the enzymic steps except chitobiase and the proteases.

Table II. Some lysosomal proteases (after Mason, 1996)

Endopeptidases
Cysteine proteases: cathepsins B*, C*, H*, K, L, O, S, and W
Aspartyl proteases: cathepsins D and E
Serine proteases: cathepsin G in azurophil granules of neutrophils
Exopeptidases
Carboxypeptidases: lysosomal carboxypeptidase (cathepsin A or protective protein)—serine protease; cathepsin B (dipeptidase); cathepsin X, mono- or dipeptidase; lysosomal carboxypeptidase B; prolylcaboxypeptidase; peptidyl dipeptidase B
Aminopeptidases: cathepsin H—true aminopeptidase; dipeptidyl peptidase I (cathepsin C); dipeptidyl peptidase II; tripeptidyl peptidase (TPP-I)

*Also have exopeptidase activity.

acidic pH, although this may not be their pH-optimum. They are synthesized in the form of inactive precursors, preproenzymes, which are transported to the lysosome by the mannose-6-phosphate pathway like other lysosomal hydrolases. The presequence is removed after leading the nascent protein into the lumen of the ER, and the prosequence is removed in the lysosome to generate the active conformation (Erickson, 1989; Ishidoh and Kominami, 2002).

Proteases are classified by the catalytic residue in the active site involved in the mechanism of peptide bond cleavage. Cathepsins with a serine (cathepsins A and G), cysteine (B, C, F, H, K, L, O, S, and W) or an aspartic acid (D and E) residue in the active site have been characterized. The specificity of a protease is determined by multiple interactions between the polypeptide substrate and amino acid residues in the active site of the enzyme (Barrett, 1994). The recent elucidation of the 3D structure of over 10 cathepsins has thrown light on the molecular basis of their mechanism of action and specificity (reviewed in Winchester, 2004). The digestion of model substrates indicates that lysosomal endopeptidases have overlapping specificities (Bromme *et al.*, 1989), suggesting flexibility in their substrate specificity pockets. This would be advantageous in dealing with the vast number of proteins degraded in lysosomes. This does not preclude preferred or resistant cleavage sites on individual substrate proteins. Many cathepsins are ubiquitously expressed, for example, cathepsins B, H, and L, but others are cell-specific, such as cathepsin S, in lymphatic tissues, where it plays an important role in processing major histocompatibility complex II complexes in antigen presentation. Cathepsin K, which has potent collagenolytic activity, is highly expressed in osteoclasts and plays an important role in digestion of the organic matrix in bone resorption in the acidified subosteoclastic space, a kind of or extracellular lysosome (Gelb *et al.*, 2001).

Lysosomal proteases along with other lysosmal enzymes can also be released into the cytosol as a result of permeabilization of the lysosome. The extent of permeabilization of the lysosomal membrane and hence the concentrations of lysosomal proteases released into the cytosol determine the level of the resultant cellular damage. Complete disruption of the membrane will lead to uncontrolled cell death or necrosis, whereas limited release can lead to apoptosis if the protective effect of endogenous inhibitors (stefins) in the cytosol is exceeded (Guiccardi *et al.*, 2004). The released proteases may act directly on substrates, including glycoproteins, or work in concert with caspases in the signaling pathways in apoptosis upstream of mitochondria. Both intra- and extralysosomal factors can lead to permeabilization of the lysosome, including sphingosine and reactive oxygen species. The lysosomal pathway of apoptosis may be especially important under pathological conditions and

contribute to the pathophysiology of some lysosomal storage diseases (Tardy *et al*., 2004).

The concentration of the endopeptidases, cathepsins B and D, in the lysosomes of liver cells is very high, ~1 mM (Dean and Barrett, 1976; Mason *et al*., 1989), emphasizing the importance of this initial step in proteolysis. The action of the endopeptidases creates more N- and C-terminal substrates for the exopeptidases, and it is probable that the endo- and exopeptidases act simultaneously. In fact several endopeptidases—for example, cathepsins B and C—also have exopeptidase activity. Exopeptidases are subdivided into carboxypeptidases and aminopeptidases (Table II). Some are specific dipeptidyl and tripeptidyl peptidases, which can cleave two or three amino acids from the C- and N-terminals. The absence of a nonspecific dipeptidase probably accounts for the presence of dipeptides among the end products of lysosomal digestion of proteins.

Lysosomal catabolism of N-linked glycoproteins

The catabolism of the N-linked glycoproteins has been studied more intensively than the digestion of other classes of glycoproteins because of the storage diseases (glycoproteinoses) resulting from defects in their catabolism. The pathways for the catabolism of complex, hybrid, and high-mannose N-linked glycans have been elucidated by the analysis of the structures of the storage products, substrate specificity studies in vitro and in vivo and the use of inhibitors, particularly amino-sugars, to block specific steps. The pathways for their breakdown are bidirectional with the sequential release of monosaccharides by exoglycosidases from the nonreducing end of the glycan and proteolysis and the digestion of the carbohydrate–polypeptide linkage at the other end (Abraham *et al*., 1983; Aronson, 1999; Baussant *et al*., 1986; Kuranda and Aronson, 1986) (Figure 3). The pathways are highly ordered and the relative rates in the two directions will depend on the accessibility of the glycosidic and peptide–glycan linkages resulting from the local conformation of the glycan and polypeptide chain.

Proteolysis and breakdown of the linkage region. The first step in this direction is proteolysis of the polypeptide as just described. The breakdown of the N-glycans is not a prerequisite for proteolysis, as is shown by the structures of the storage products in the glycoproteinoses (Abraham *et al*., 1983; Thomas, 2001). Storage products consisting of intact glycans or glycans only partially degraded at the nonreducing end occur either as free oligosaccharides or linked to single asparagine residues, indicating that the polypeptide can be broken down without complete degradation of the oligosaccharide. The low activity of purified lysosomal glycosidases on intact N-glycoproteins, albeit in vitro, suggests that the polypeptide has to be denatured or broken down before the glycan can be degraded. This is supported by the specificity of glycosylasparaginase, the enzyme that cleaves the protein–carbohydrate linkage, which requires the asparagine to have free α-amino and α-carboxyl groups (Aronson, 1999). These observations suggest that complete or extensive proteolysis takes place before the N-linked glycan is released from the polypeptide.

The next step in the breakdown of complex or hybrid N-glycans is the removal of any fucose residues, which is catalyzed by lysosomal α-L-fucosidase. Fucose can be linked α1→6 linked to the core N-acetylglucosamine attached to the asparagine, α1→2, 3, or 4 to N-acetylglucosamine or α1→2 to galactose residues at the nonreducing end(s) of the glycan (Johnson and Alhadeff, 1991). The urinary storage products in human and canine fucosidosis contain a high proportion of glycoasparagines with a fucose α1→6 linked to the core chitobiose (Figure 4) (Barker *et al*., 1988; Tsay *et al*., 1976; Yamashita *et al*., 1979). This can be explained by steric hindrance of the glycosylasparaginase by the core α1→6 linked fucose (Barker *et al*., 1988; Noronkosi and Mononen, 1997; Tarentino *et al*., 1975; Yamashita *et al*., 1979). Thus the storage products in fucosidosis consist of fucosylated glycoasparagines and oligosaccharides without a core α1→6 linked fucose (Figure 4). Interestingly analysis of the digestion of the canine storage products by purified α-L-fucosidase indicated that the peripheral α1→3 linked fucose residues were removed before the core α1→6 linked

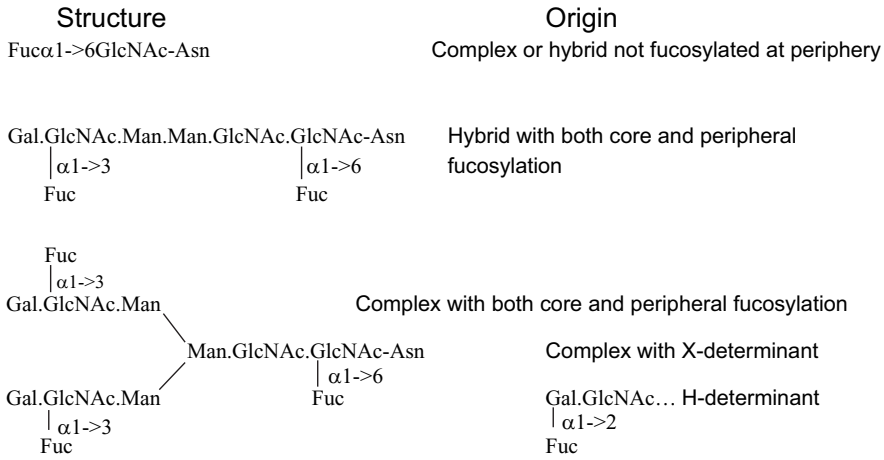

Fig. 4. Structures of some storage products in fucosidosis. Fucosidosis results from a deficiency of lysosomal α-L-fucosidase and has been found in humans and dogs.

fucose residues (Barker *et al*., 1988). This suggests that α-L-fucosidase plays an important role in controlling the bidirectional catabolism of fucosylated glycans.

Removal of fucose allows the hydrolysis of the asparagine-*N*-acetylglucosamine amide linkage by glycosylasparaginase (aspartamidohydrolase) as long as there are free α-amino and α-carboxyl groups on the asparagine (Aronson, 1999; Kaartinen *et al*., 1992; Tanaka *et al*., 1973). This enzyme is a member of the N-terminal nucleophile (Ntn) hydrolases, and its 3D structure is known (Oinonen *et al*., 1995). It undergoes dimerization and autoactivation by cleavage into two subunits in the Golgi. Many of the disease-causing mutations found in patients with aspartylglucosaminuria prevent correct folding of the enzyme and activation, including the common Finnish genotype (Saarela *et al*., 2001).

The release of the *N*-acetylglucosamine residue at the reducing end of the chitobiose core can be achieved in two ways, depending on the species and the relative rates of the two sides of the bidirectional pathway. An endo-β-N-acetylglucosaminidase or lysosomal di-*N*-chitobiobiase is present in humans and rodents (Kuranda and Aronson, 1986; Stirling, 1974). It acts as a reducing end exohexosaminidase to remove a single *N*-acetylglucosamine from the reducing end of glycans. This enzyme is not expressed in ungulates or cats and dogs and explains the difference in the structures of the storage products in the glycoproteinoses between these groups of species. In humans and rodents there is a single *N*-acetylglucosamine at the reducing end, whereas di-N-acetylchitobiose is present in cattle, pigs, sheep, cats, and dogs (Abraham *et al*., 1983). In those species lacking the lysosomal di-*N*-chitobiobiase, the reducing end *N*-acetylglucosamine has to be removed by the exoglycosidase, β-N-acetylglucosaminidase, as the last step in the breakdown. In humans and rodents the *N*-acetylglucosamine can be removed by either the β-N-acetylglucosaminidase or the di-*N*-acetyl-chitobiobiase. Comparison of the structures of the human and bovine genes offers an intriguing explanation of the difference in the expression of this enzyme in these species. The 5′-flanking region of the human gene has typical features of a housekeeping gene, whereas the same region of the bovine gene has several potential silencers (Liu *et al*., 1999).

Breakdown of oligosaccharide from nonreducing end. N-linked glycans are broken down from the nonreducing end by the sequential release of monosaccharides catalyzed by lysosomal exoglycosidases, as is shown in Figure 3 for a typical complex glycan. The exoglycosidases are specific for the nonreducing end of a glycosyl structure and act in a sequence determined by the structure of the glycan. Some of the glycosidases—for example, α-neuraminidase, β-galactosidase, and N-acetyl-β-D-hexosaminidase—also catalyze the same linkages in other glycoconjugates, such as glycolipids or glycosaminoglycans, whereas α-mannosidase and β-mannosidase appear to be exclusive to the glycoprotein catabolic pathway so far. All of these enzymes have been cloned and mutations in the genes shown to be responsible for the different glycoproteinoses (Table I). The structures of the storage products derived from complex N-linked glycans in the glycoproteinoses are consistent with an obligatory order of breakdown of the branches by single gene products. It is not known whether the different branches of bi- and triantennary glycans are broken down at different rates.

The structures of the oligosaccharide storage products that accumulate in α-mannosidosis due to a deficiency of lysosomal α-D mannosidase confirm that mature glycoproteins containing high-mannose and hybrid glycans are also turned over in lysosomes (Figure 5) (Yamashita *et al*., 1980). The pathways for the breakdown of high-mannose and hybrid glycans by human lysosomal α-mannosidase have also been elucidated (Al Daher *et al*., 1991) (Figure 6). The digestion of $Man_9GlcNAc$ is an ordered process with a

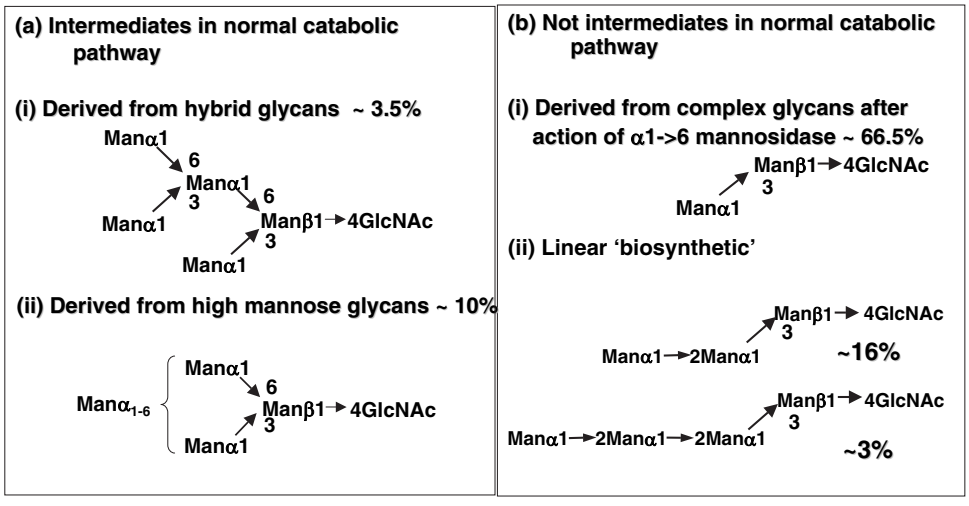

Fig. 5. Structures of urinary storage products in human α-mannosidosis. (**a**) Structures found as intermediates in normal catabolic pathway (i) and (ii) derived from breakdown of hybrid and high mannose N-linked glycans, respectively. (**b**) Structures not found in normal catabolic pathway (i) derived from core structure of complex N-linked glycans after action of lysosomal α-1–6-mannosidase (ii) linear structures resembling intermediates in biosynthetic pathway.

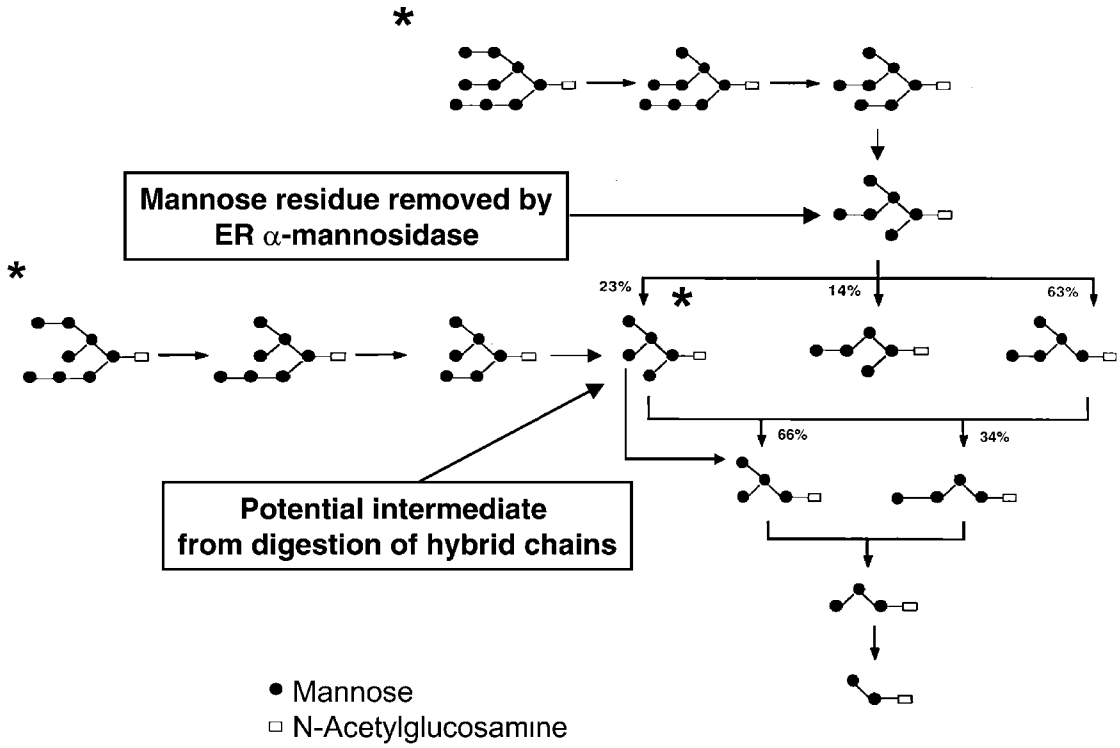

Fig. 6. Digestion in vitro of high-mannose and hybrid glycans by purified human lysosomal α-D-mannosidase. Purified oligosaccharides* were digested with purified human lysosomal α-D-mannosidase and products analyzed by high-performance liquid chromatography.
*Expected to accumulate in α-mannosidosis due to deficiency of lysosomal α-D-mannosidase.

unique route to $Man_6GlcNAc$ involving hydrolysis of three specific α1→2mannosidic linkages. Interestingly the α1→2-linked mannose residue normally removed by the ER α-mannosidase I (Bischoff *et al.*, 1986) is very resistant to hydrolysis by lysosomal α-mannosidase. This suggests that this mannosyl residue is not common in the high-mannose structures found on mature glycoproteins delivered to lysosomes. Direct digestion in vitro of the $Man_8GlcNAc$ structure produced by the ER α-mannosidase I (Figure 6) confirms the pathway. Once exposed the core α1→3 mannosidic linkage is very vulnerable to hydrolysis, and the core tetrasaccharide structure is not an intermediate in the pathway. The core α1→6 mannosidic linkage is the most resistant to hydrolysis.

The $Man_5GlcNAc$ digestion intermediate in the catabolism of high-mannose glycans could also be an intermediate in the lysosomal digestion of hybrid glycans. Digestion of this intermediate with purified enzyme followed the same pathway, as observed for the same structure in the digestion of high-mannose glycans (Figure 6). The relative rates of lysosomal catabolism of the complex and high-mannose branches of a hybrid glycan are not known but the structures of the storage products in the glycoproteinoses suggest that the digestion of the two branches is independent. Thus, partially digested complex branches linked α1→3 to the core β-linked mannose with no α1→6 mannose or polymannose glycan attached are found in sialidosis, β-galactosidosis, and Sandhoff disease (Thomas, 2001). This would also explain the occurrence of the $Man_5GlcNAc$ in α-mannosidosis. The hybrid intermediate with two N-acetylglucosamine residues at the reducing end is broken down by the human enzyme by the same route but the core α1→6-mannosidic linkages are again very resistant to hydrolysis. The pathway in rats is similar (Michalski *et al.*, 1990). The pathways for the breakdown of high-mannose and hybrid glycans with two N-acetylglucosamine residues at the reducing end by bovine and feline lysosomal α-mannosidases are similar but show subtle differences (De Gasperi *et al.*, 1991). It is concluded that the specificity of each step in the breakdown of high-mannose glycans by lysosomal α-mannosidase is determined by the conformation of the oligosaccharide in the active site of the enzyme, with the α-mannosidic linkage in juxtaposition to the catalytic groups being the most vulnerable to hydrolysis (Daniel *et al.*, 1994).

The core structure common to all *N*-linked-glycans, $Man\alpha1\rightarrow3[Man\alpha1\rightarrow6]\ Man\beta1\rightarrow4GlcNAc_2$ is not an intermediate in the breakdown of high-mannose or complex glycans. Although purified human lysosomal α-mannosidase can hydrolyze α1→2, α1→3, and α1→6 mannosidic linkages, there is evidence that a second lysosomal α-mannosidase participates in the digestion of the core region of N-linked glycans (Al Daher *et al.*, 1991; Cenci di Bello *et al.*, 1983; Daniel *et al.*, 1992). In vitro digestion of the core pentasaccharide $Man_3GlcNac_2$ or tetrasaccharide $Man_3GlcNAC$ with purified human lysosomal α-mannosidase showed that the core α1→3 mannosidic linkage is cleaved very readily but the α1→6 mannosidic linkage only very slowly, in fact hardly at all if the chitobiose linkage is intact (Al Daher *et al.*, 1991) (Figure 7). This suggests either that there is a distinct lysosomal α1→6 mannosidase in intact human lysosomes or that these structures do not occur in the

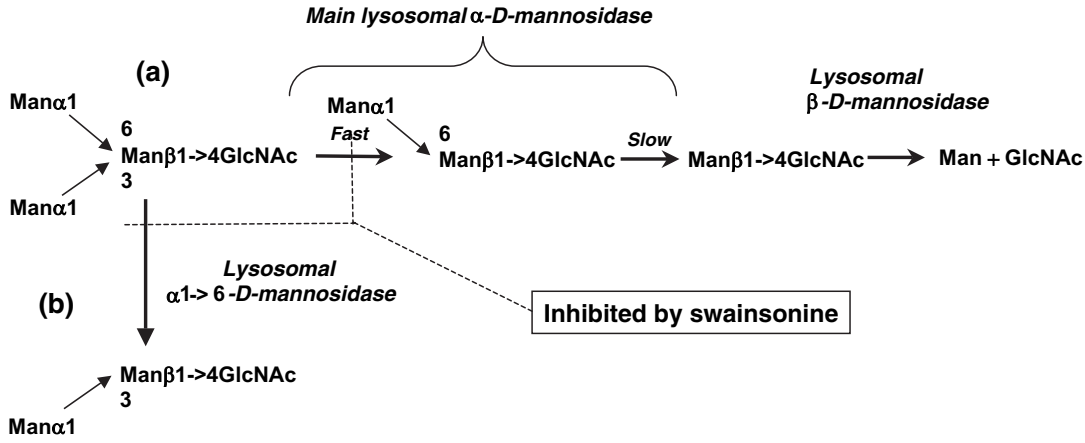

Fig. 7. Digestion of the core structure derived from N-glycans by human lysosomal α-D-mannosidases and β-D-mannosidase. (**a**) Normal pathway for catabolism of Manα1→3[Manα1→6]Manβ1→4GlcNAc. (**b**) Pathway in genetic α-mannosidosis leading to accumulation of Manα1→3Manβ1→4GlcNAc. Swainsonine inhibits both the main lysosomal α-D-mannosidase and the α1→ -D-mannosidase resulting in the accumulation of Manα1→3[Manα1→6]Manβ1→4GlcNAc.

catabolic pathway. Indirect evidence for the existence of the lysosomal α1→6 mannosidase comes from the structures of the major storage products in human α-mannosidosis, all of which retain the core α1→3 mannosidic linkage but not the core α1→6 mannosidic linkage (Yamashita *et al*., 1980) (Figure 5). This indicates that in the absence of the major lysosomal α-mannosidase the α1→6-mannosidic linkage is cleaved by another activity. The α1→6 mannosidase has been isolated and characterized (Daniel *et al*., 1992; De Gasperi *et al*., 1992) (Figure 7). It requires the prior removal of the *N*-acetylglucosamine at the reducing end of the chitobiose structure, explaining the specificity studies (Haeuw *et al*., 1994). It is only expressed in tissues and species expressing the lysososmal chitobiase, suggesting that the two activities are functionally linked (Daniel *et al*., 1994). Its presence explains the structure of the main storage product derived from complex N-linked glycans in human α-mannosidosis (Figure 7).

The final step in the catabolism of the N-linked oligosaccharide in humans and rodents is catalyzed by β-mannosidase (Figures 3 and 7). The deficiency of β-mannosidase in β-mannosidosis (Cooper *et al*., 1986; Wenger *et al*., 1986) leads to the accumulation of Manβ1→4GlcNAc together with its sialylated derivative and a urea conjugate, which probably arise from secondary reactions with primary storage product (Dorland *et al*., 1988). The β-mannosidase gene has been cloned, and mutations have been identified in a few of the patients with this very rare and relatively mild disease (Alkhayat *et al*., 1998). If the rate of digestion of the oligosaccharide chain is faster than the degradation of the protein–carbohydrate linkage region, then the first N-acetylglucosamine residue of the di-*N*, *N*′-acetylchitobiose may be removed by the exoglycosidase, *N*-acetyl-β-D-glucosaminidase rather than the lysosomal chitobiase. In species that do not express lysosomal chitobiase, such as ungulates, this is the only way of removing this residue. β-Mannosidosis occurs in goats (Jones and Dawson, 1981) and cattle (Bryan *et al*., 1990), and the storage products terminate in di-*N*,*N*′-acetylchitobiose at the reducing end, consistent with the absence of lysosomal chitobiase.

It is important to note that the intermediates in the lysosomal catabolism of complex, high-mannose, and hybrid N-linked glycans are distinct from the intermediates in the biosynthetic pathway.

Lysosomal catabolism of O-linked glycoproteins

Several different types of O-glycosylation occur in mammals, including the attachment of (1) glycosaminoglycans by a xylosyl-serine linkage; (2) short mucin-type oligosaccharide chains by *N*-acetylgalactosamine (GalNAc) to serine or threonine; (3) mannose, fucose, and *N*-acetylglucosamine to serine/threonine; and (4) the disaccharide glucosylα1→2galactose to 4-hydroxylysine residues in collagen and related molecules. The attachment of a single *N*-acetylglucosamine to serine/threonine in nuclear and cytoskeletal proteins is transient, with its removal catalyzed by a specific cytosolic *N*-acetylglucosaminidase (Hart, 1997). It is presumed that all the other types of O-glycosylated proteins are turned over in the lysosomes after delivery by endocyosis or autophagy. Cathepsin K has been shown to be essential for the intracellular degradation of collagen fibrils in synovial fibroblasts (Hou *et al*., 2001) and to be upregulated in lung fibroblasts from patients with fibrosis (Bühling *et al*., 2004) Relatively little has been published on the enzymology of the lysosomal catabolism of O-linked glycans, with the exception of the glycosaminoglycans of proteoglycans (Neufeld and Muenzer, 2001). It is probable that the same lysosomal enzymes catalyze the hydrolysis of the same glycosidic linkages in O-linked glycans as in other glycoconjugates. For example, the lysosomal β-galactosidase acts on a wide range of β-galactosidic linkages in glycosphingolipids, N-linked glycans, and glycosaminoglycans. Similarly, the exoglycosidases β-*N*-acetylglucosaminidase, sialidase, and α-fucosidase are probably involved in the lysosomal pathway for the catabolism of O-linked glycans, although the concentrations of oligosaccharides that might arise from the incomplete catabolism of O-linked glycans are not prominent in the urines of patients with deficiencies of some of these enzymes. This may reflect the relative abundance

of N- and O-linked glycans or the catabolic pathway. For example, it is not known whether the O-glycan has to be released from the protein for degradation by exoglycosidases or whether extensive proteolysis is a prerequisite for the catabolism of O-linked glycans. The release of glycosaminoglycans linked by β-xylosyl-Ser/Thr linkage to the core proteins of proteoglycans is catalyzed by a specific lysosomal endo-β-xylosidase (Takagaki *et al*., 1988). In contrast, the multifunctional exoglycosidase α-*N*-acetylgalactosaminidase can cleave the α-*N*-acetylgalactosaminyl–Ser/Thr linkage of the mucin-type O-glycans. The α-mannosidase activity that hydrolyzes the α-mannosyl-Ser/Thr linkage of O-mannosylated proteins has not been characterized. It could be the major, broad specificity α-mannosidase, the minor lysosomal α1→6 mannosidase, or an as yet unknown activity. Similarly it is not known whether a specific α-fucosidase cleaves the fucosyl Ser/Thr linkage.

The most common form of O-glycosylation is the mucin type, with the α-*N*-acetylgalactosamine–Ser/Thr linkage. The mucin type glycans are subdivided into core structures on the basis of the second sugar(s) and/or sugar binding. The core 1 mucin type O-linked glycan occurs on many membrane and secreted proteins, especially in brain and neural tissue, and is the precursor of the branched core 2 structure. A deficiency of α-*N*-acetylgalactosaminidase (also known as α-galactosidase B) leads to the rare lysosomal storage disease Schindler or Kanzaki disease (Desnick and Schindler, 2001), in which there is accumulation of sialylated and asialo-mucin type glycopeptides as well as oligosaccharides and probably blood group A glycosphingolipids with α-*N*-acetylgalactosaminyl residues at the nonreducing end (Figure 8). The expected major storage product from the catabolism of mucin type O-glycans, GalNacα1→Ser/Thr, is found in the urine and is the only storage product detectable by immunoelectron microscopy in lysosomes of skin cells from patients (Kanda *et al*., 2002). However, there are also large amounts of mucin type O-glycopeptides in the urine, suggesting that the O-glycans cannot be broken down by exoglycosidases until they are released from the peptide. This in turn implies that α-*N*-acetylgalactosaminidase has endo-α-N-acetylgalactosaminidase, comparable to the lysosomal di-*N*-chitobiobiase in N-glycan catabolism. It is also possible that the longer glycopeptides arise from transglycosylation of the primary storage product, GalNAcα1→Ser/Thr, as occurs in β-mannosidosis (van Pelt *et al*., 1990). A fascinating alternative explanation is based on the observation that the structures of the sialylglycopeptides found in Schindler disease are very similar to those found in the urine of patients with sialidosis (Lecat *et al*., 1984) and galactosialidosis (Takahashi *et al*., 1991). These disorders result from an isolated deficiency of sialidase and a combined deficiency of sialidase and β-galactosidase due to mutations in the protective protein (cathepsin A), respectively. This suggests that the multienzyme complex containing β-galactosidase, sialidase, and the protective protein (d'Azzo, 2004) can also interact with α-*N*-acetylgalactosaminidase (Desnick and Schindler, 2001) to form a larger functional complex for the ordered breakdown of sialylated mucin type O-glycans. Consequently a defect in any one of the constituent enzymes could disrupt this pathway and lead to the accumulation of common sialoglycopeptides. The multienzyme complex has been shown to associate, perhaps transiently, with at least one other lysosomal enzyme, N-acetylgalactosamine-6-sulfate sulphatase (Pszezhetsky and Potier, 1996). The formation of transient complexes of all the lysosomal enzymes in the pathway for the catabolism of a particular class of substrates is an attractive hypothesis. The accumulation of intermediates in such complex could lead to efficient transglycosylation or inhibition of enzymes with close substrate specificities and consequent production of storage products with unexpected structures.

The occurrence of the blood group A trisaccharide, GalNAcα1→3[Fucα1→2]Gal, in the urine of group A–positive patients with Schindler disease implies the action of an endo-β-galactosidase in the catabolism of N-linked glycopeptides and glycosphingolipids carrying the blood group A structure. This may be a minor pathway only detected in the absence of the exoglycosidase α-*N*-acetylgalactosaminidase.

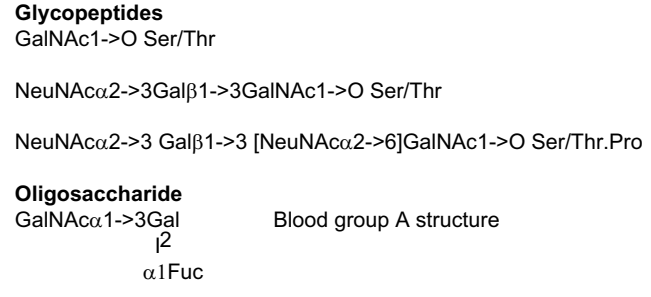

Fig. 8. Structures of some storage products in Schindler (Kawasaki) disease due to a deficiency of α-*N*-acetylglucosaminidase.

Lysosomal catabolism of GPI-anchored proteins

There is very little information on the involvement of lysosomes in the turnover of GPI-anchored proteins (Mann *et al*., 2004). GPI-hydrolyzing phospholipases (C and D) have been described in many mammalian tissues and fluids (Davitz *et al*., 1987). A GPI-specific phospholipase D (GPI-LPD) has been colocalized with typical lysosomal hydrolases in rat liver, suggesting that the turnover of GPI anchors takes place at least partly in the lysosome (Hari *et al*., 1996). The circulating GPI-LPD enzyme does not cleave GPI-anchored proteins on cell surfaces, but it may be taken up into cells and delivered to the lysosome (Hari *et al*., 1996). GPI-LPD has also been localized to ER membranes and isolated lipid rafts, suggesting it may control the level of GPI anchors by cleaving GPI intermediates intracellularly (Mann *et al*., 2004). Thus proteins with an inositol-containing glycan attached via phosphoethanolamine could be generated in the lysosomes or delivered to the lysosomes by autophagy or endocytosis after formation elsewhwere. The protein and glycan moieties could then be broken down by the lysosomal enzymes.

Transport of digestion products out of lysosome

Amino acids and peptides

The lysosomal proteolysis of polypeptides results in a mixture of amino acids and a few dipeptides, which are transported

out of the lysosome into the cytosol for reuse by the cell by a combination of passive diffusion and specific transporters (Lloyd, 1996; Mancini *et al*., 2000; Winchester, 2001). The ability of small molecules to diffuse through the lysosomal membrane has been predicted to be inversely proportional to hydrogen-bonding capacity (Lloyd, 1996). Therefore, passive diffusion probably only occurs for the small, neutral, zwitterionic amino acids, such as alanine and glycine, and is supplemented by specific transport. At least 10 distinct lysosomal membrane transporters for amino acids have been detected biochemically in human cells (Lloyd, 1996; Pisoni and Thoene, 1991; Sagné *et al*., 2001). Collectively they can account for the transport of all amino acids except asparagine and glutamine. They have low K_m values of 10–320 μM, which are similar to the concentrations of amino acids reported in lysosomes (Harms *et al*., 1981; Vadgama and Jonas, 1992). Consequently, small changes in the concentrations of amino acids will have a rapid effect on the efflux rate.

The only amino acid transporters to be fully characterized by cloning are the cystine transporter cystinosin (Town *et al*., 1998) and LYAAT-1, the transporter for small neutral amino acids (Aguhlon *et al*., 2003). Mutations in the cystinosin gene (*CTNS*) lead to the disorder cystinosis, in which there is massive intralysosomal accumulation of free cystine. The deposition of cystine crystals in the kidney led to progressive renal failure and death in the first decade of life before therapy with cysteamine became available (Gahl *et al*., 1987). Cysteamine can pass through the plasma and lysosomal membranes at physiological pH but becomes trapped by protonation in the lysosomes. There it reacts with the accumulated cystine to form cysteine and a mixed disulfide, cyteamine-cysteine, which resembles lysine structurally and is transported out of the lysosomes by the lysine transporter. The cysteine produced is transported out of the lysosomes by the cysteine transporter, which is distinct from the cystine transporter and is unaffected in cystinosis. The severity of cystinosis illustrates the importance of the integrity of the whole lysosomal system, not just enzyme activity. It also shows how knowledge of the lysosomal system can be exploited for therapy.

Indirect evidence for an oligopeptide transporter in rat liver lysosomes has been obtained by studying the uptake of the dipeptide, Gly-Gln (Thamotharan *et al*., 1997; Zhou *et al*., 2000). Gly-Gln is taken up into the lysosomes with a low affinity (K_m 5–10 mM) in a proton-coupled process, which can be inhibited by other di- and tripeptides but not by amino acids. A novel rat peptide/histidine transporter with specificity for histidine and histidyl leucine has been cloned and shown to be localized especially in the lysosomal membrane of lymphatic cells (Sakata *et al*., 2001). These transporters would provide a mechanism for transporting the dipeptides produced in lysosomal proteolysis from the low dipeptidase activity of the lumen of the lysosome to the high dipeptidase activity in the cytosol, thereby completing the catabolism of proteins.

Sugars

Three lysosomal monosaccharide transporters have been described, based on the kinetics of uptake or release of monosaccharides from isolated lysosomes (Lloyd, 1996). They are specific for neutral hexoses, *N*-acetylhexosamines, and acidic monosaccharides (iduronic, glucuronic, and sialic acids), respectively. Collectively their specificities cover all 11 monosaccharides produced in mammalian lysosomes by the digestion of glycoconjugates. The gene for the sialic acid transporter, *AST*, has been isolated by positional cloning (Verheijen *et al*., 1999) and shown to be mutated in patients with sialic acid storage disease. The predicted protein, sialin, has 495 amino acids with 12 transmembrane domains and 6 potential N-glycosylation sites, but no signal peptide or known lysosomal targeting signal. However, sialin has been localized to lysosomes in mammalian cells in culture (Aula *et al*., 2002). In primary neuronal cultures, it is located in the processes and plasma membrane, suggesting an additional extralysosomal role in secretion in neurones (Aula *et al*., 2004). Sialin shows 37% and 61% identity and similarity, respectively, to a human brain-specific Na^+–phosphate symporter. It contains a motif in the fourth transmembrane domain characteristic of anion/cation symporters, indicating that it is probably a member of the major facilitator superfamily of transporters. The severity of sialic storage disease illustrates the importance of clearing the monosaccharide digestion products from the lysosome. The incorporation of radiolabeled sialic acids released by lysosomal catabolim of labeled glycoconjugates into newly synthesized gangliosides and sialoglycoproteins is greatly impaired in sialin-deficient fibroblasts (Chigorno *et al*., 1996). This provides direct evidence for the reutilisation of lysosomal digestion products by cells and confirms many early studies of the turnover of labeled endogenous glycoproteins.

Relationship between nonlysosomal and lysosomal catabolism of N-linked glycoproteins

Although the physiological function of most lysosomal membrane transporters appears to be the export of digestion products from the lysosome, the free oligosaccharide transporter may be an example of a specific import mechanism (Saint-Pol *et al*., 1999). Free oligosaccharides in the cytosol are imported into the lysosome by the ATP-dependent, high-affinity oligosaccharide transporter (Moore, 1999) and degraded. Free oligosaccharides arise in the cytosol as a result of breakdown of lipid-linked oligosaccharides and from glycopeptides and incorrectly folded glycoproteins generated during quality control analysis of the biosynthesis of glycoproteins in the ER (Spiro, 2004) (Figure 9). Free oligosaccharides with di-*N*-acetylchitobiose at the reducing end can be produced in the lumen of the ER by the hydrolytic action of the oligosaccharyltransferase (Spiro and Spiro, 1991). They are subsequently transported into the cytosol (Moore *et al*., 1995) where they are acted on by a neutral cytosolic chitobiase to form oligosaccharides with a single N-acetylglucosamine residue at the nonreducing end (Cacan *et al*., 1996). Four mannose residues are then removed by neutral cytosolic α-mannosidase to produce a specific limit digestion product, Manα1→2Manα1→2Manα1→3[Manα1→6]Manβ1→4GlcNAc in quail (Oku and Hase, 1991), rat (Haeuw *et al*., 1991), humans (Al Daher

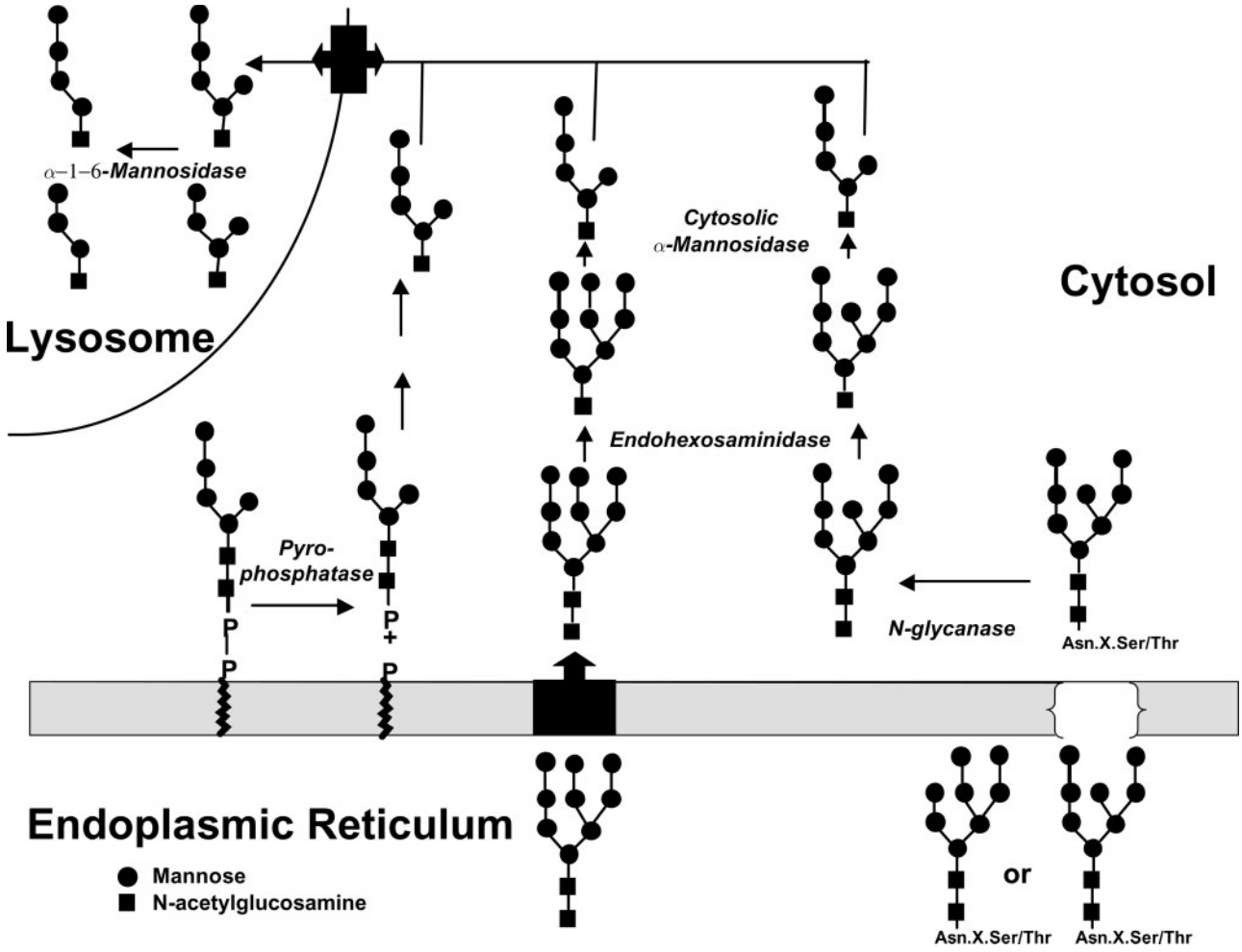

Fig. 9. Relationship between editing of N-glycosylation in ER and Golgi apparatus and cytosolic and lysosomal catabolism of aberrant glycoproteins.

et al., 1992), and cattle and cats (De Gasperi *et al*., 1992) (Figure 10).

The same specific oligosaccharide can be produced in the cytosol by two other routes. Phosphorylated $Man_5GlcNAc_2$ and possibly $Man_2GlcNAc_2$ can be released from dolichol by the action of pyrophosphatase at the cyosolic face of the ER (Cacan *et al*., 1992). Removal of the phosphate from the $Man_5GlcNAc_2$ generates the oligosaccharide with the same structure as the limit product of the cytosolic α-mannosidase. Second, misfolded glycoproteins can be exported from the ER into the cytosol via a channel containing Sec61 (Cacan *et al*., 2001), where they are acted on by a cytosolic endoglucoasminidase or peptide:N-glycanase (Hirsch *et al*., 2003; Pierce *et al*., 1979). This enzyme is probably part of a complex involved in the ubiquitin/proteasome degradative pathway (Suzuki and Lennarz, 2003). The released glycan is acted on successively by the cytosolic chitobiase and neutral α-mannosidase to form the limit digestion product. The final step in the cytosolic pathway is the transport of the limit digestion oligosaccharide into lysosomes by the by the ATP-dependent, high-affinity oligosaccharide transporter (Moore, 1999; Saint-Pol *et al*., 1999).

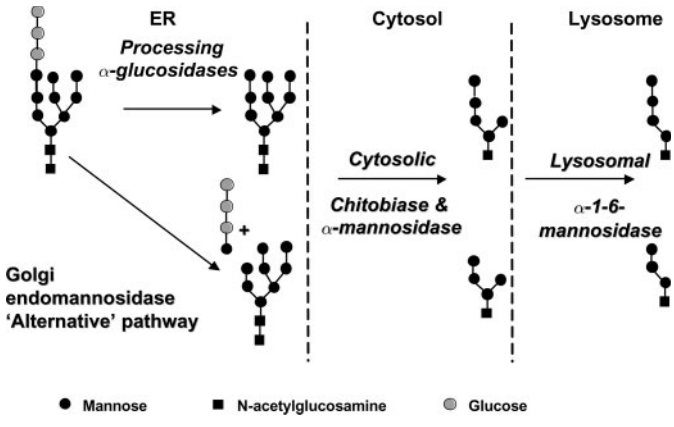

Fig. 10. Postulated origin of linear biosynthetic storage products in α-mannosidosis.

The existence of the nonlysosomal pathway for the degradation of misfolded glycoproteins and surplus key lipid-linked oligosaccharide intermediates explains the structures

of the anomalous linear storage products in human α-mannosidosis (Figure 5). The linear storage products, (Manα1→2) Manα1→2 and Manα1→3Manβ1→4GlcNAc, are not intermediates in the lysosomal pathway for the breakdown of the high-mannose, hybrid, and complex N-linked glycans found on mature glycoproteins (Al Daher *et al*., 1991). Manα1→2Manα1→2Manα1→3Manβ1→4GlcNAc can be derived from the limit digestion product of the cytosolic pathway, Manα1→2Manα1→2Manα1→3[Manα1→6] Manβ1→4GlcNAc, by the removal of the α1→6 linked mannose residue catalyzed by the α1→6 mannosidase (Cenci di Bello *et al*., 1983; Daniel *et al*., 1994) (Figure 10). Manα1→2Manα1→3Manβ1→4GlcNAc may also be derived from partially processed glycoproteins by the successive action of the Golgi endomannosidase (Lubas and Spiro, 1987), the cytosolic enzymes, and the lysosomal α1→6 mannosidase (Figure 10).

These linear biosynthetic storage products constitute ~20% of the urinary storage products in the urine of a patient with α-mannosidosis (Yamashita *et al*., 1980). This suggests that catabolism of immature glycoproteins and biosynthetic intermediates accounts for ~20% of the load of glycoproteins presented to lysosomes in human cells (Daniel *et al*., 1994). This pathway also explains the presence in the urine and tissues of cats and cattle with α-mannosidosis of storage products with a single *N*-acetylglucosamine at the nonreducing end, despite the absence of a lysosomal chitobiase (Daniel *et al*., 1994). The proportions of the storage products with a single *N*-acetylglucosamine at the nonreducing end can also be used to measure the cytosolic pathway in different tissues in these species. Similarly, the relative contributions of the lysosomal and cytosolic pathways can also be estimated from the ratio of the storage products, Manβ1→4GlcNAcβ1→4GlcNAc to Manβ1→4GlcNAc in caprine β-mannosidosis (Daniel *et al*., 1994; Matsuura *et al*., 1981).

The cytosolic/lysosomal pathway would be expected to be up-regulated under conditions in which the production of incorrectly folded or glycosylated glycoproteins is increased, for example, in patients with congenital disorders of glycosylation type I or in cells or tissues secreting high concentrations of glycoproteins according to physiological demand, such as the pancreas. In support of this hypothesis, the pancreas of cattle with genetic or swainsonine-induced α-mannosidosis contains very high concentrations of storage products with a single *N*-acetylglucosamine at the nonreducing end (Daniel *et al*., 1989). It is clear that the cytosolic/ER pathway for the catabolism of glycoproteins is an integral part of the unfolded protein response and plays an important role in the regulation of glycosylation.

Conclusion

Glycoproteins with many different functions and cellular locations are normally turned over in lysosomes and the digestion products recycled. In conjunction with the proteasome, lysosomes contribute to the regulation of synthesis of N-linked glycoproteins. Defects in the lysosomal system disrupt these processes and lead to severe diseases, illustrating the importance of the continuous cycle of biosynthesis and degradation of glycoconjugates to cellular function and adaptability. In the extreme lysosomes are involved in both necrosis and apoptosis of cells.

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Abbreviations

ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; LPD, phospholipase D.

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