

POSTTRANSLATIONAL MODIFICATIONS AND
INTRACELLULAR TRANSPORT OF LYSOSOMAL
 α -GLUCOSIDASE AND SUCRASE-ISOMALTASE

Posttranslationele modificaties en intracellulair transport
van lysosomaal α -glucosidase en sucrase-isomaltase

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*Voor mijn ouders en oma
In dankbare herinnering
aan L. Visser*

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Scope of the thesis

A characteristic feature of eukaryotic cells is compartmentation. Organelles of different structure and composition, and with a distinct function and content are limited by membranes. Metabolites, macromolecular compounds and proteins are targeted to the organelles. Intracellular transport of proteins is often accompanied by posttranslational modifications. In this thesis the attention is focused on the mechanism of intracellular transport of the lysosomal enzyme acid α -glucosidase and the posttranslational modifications that occur. A comparison is made with the structurally and functionally homologous enzymes sucrase and isomaltase which are targeted as a complex to the apical cell surface of intestinal epithelial cells. The studies were performed to increase the knowledge on molecular signals for protein targeting and to provide the basis for understanding the consequences of the various inherited mutations in the lysosomal α -glucosidase gene that lead to aberrant posttranslational processing and transport of the encoded enzyme and as a consequence to the pathological condition known as glycogen storage disease type II.

Chapter 1

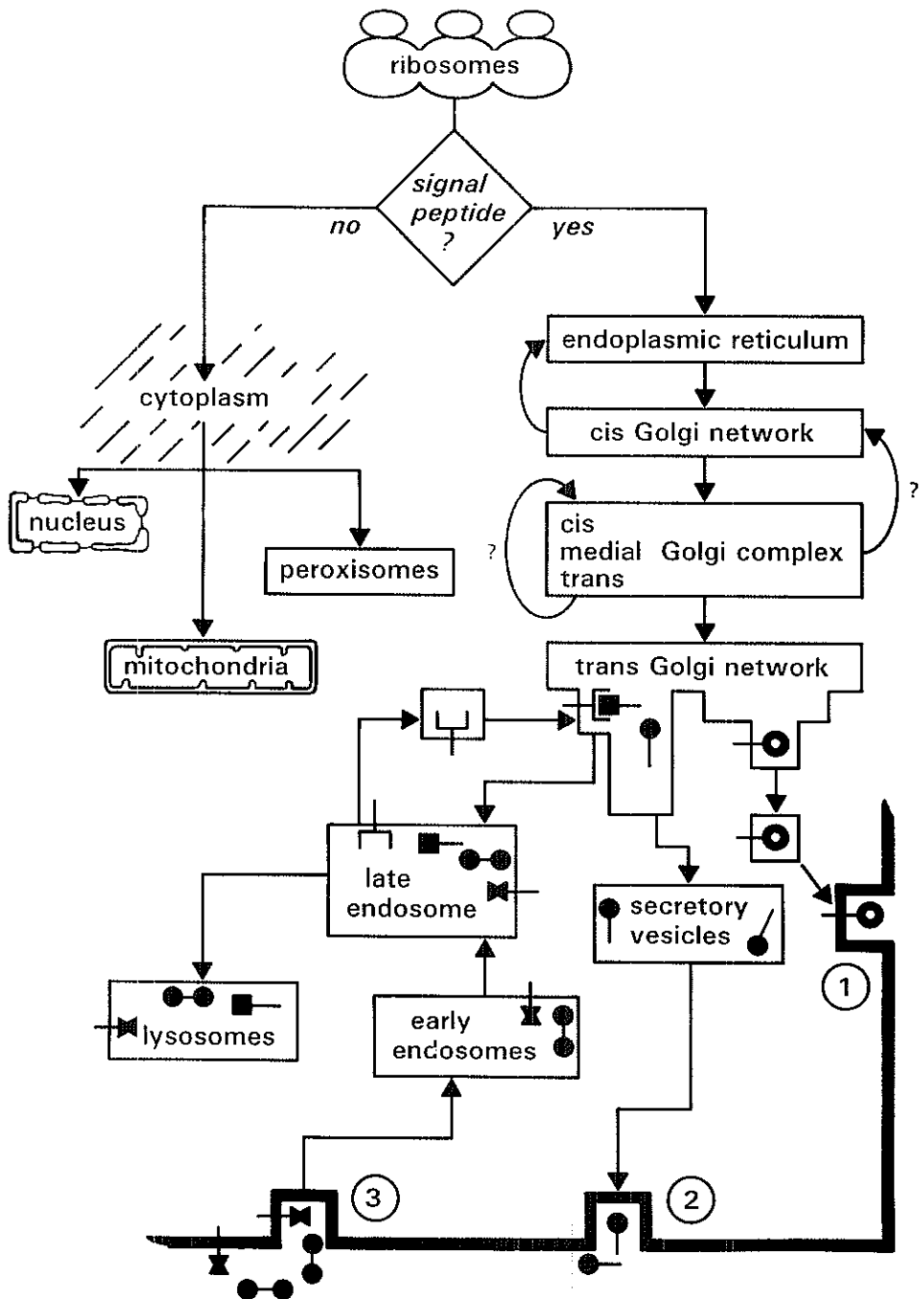
Biosynthesis and intracellular transport of glycoproteins

Since eukaryotic cells have membrane bound compartments with specialized functions (organelles) it is essential that newly synthesized proteins have build-in information to reach and enter the compartment where their specific function is required. This information is encoded in the nucleotide sequence of the DNA and via mRNA translated into the primary, or higher structures of the proteins. A simplified scheme of protein sorting is presented in figure 1. Proteins with an amino-terminal signal peptide are segregated co-translationally in the lumen of the endoplasmic reticulum and are channelled from there to their final destination. Among the latter group of proteins are the resident proteins of the endoplasmic reticulum, the Golgi complex, the trans Golgi network, the lysosomes, the endosomes and the plasma membrane as well as the secretory proteins (right branch). Proteins without an amino-terminal signal peptide for entry into the lumen of the endoplasmic reticulum end up in the cytoplasm and either stay there or are guided by signal sequences to their appropriate compartments such as the nucleus, the mitochondria and the peroxisomes (left branch).

The studies described in this thesis are focused on the transport and post-translational modification of the enzymes acid α -glucosidase and sucrase-isomaltase. Acid α -glucosidase is a typical lysosomal household enzyme with as main function the degradation of glycogen, but is in some cell types also localized at the plasma membrane. Sucrase-isomaltase has a tissue specific expression and is primarily located at the apical cell surface of intestinal epithelial cells, but is also found in lysosomes.

1.1 Protein transport across the endoplasmic reticulum membrane

For a specific group of polypeptides, the glycoproteins, the amino-terminal end serves to initiate translocation across the endoplasmic reticulum (ER) membrane. This part of the protein is known as the signal sequence or signal peptide (SP). Comparison of several known signal sequences revealed that they are composed of three different regions, an amino-terminal region (1 to 20 amino acids) with a positive net charge, a hydrophobic middle region containing 7 to 16 mainly hydrophobic amino acids and a distal region consisting of 4 to 6 relatively polar residues (Von Heijne, 1983; 1985; 1986, 1988; Gierasch, 1989). The signal peptide is recognized and bound by a protein complex known as the signal recognition particle (SRP; Walter et



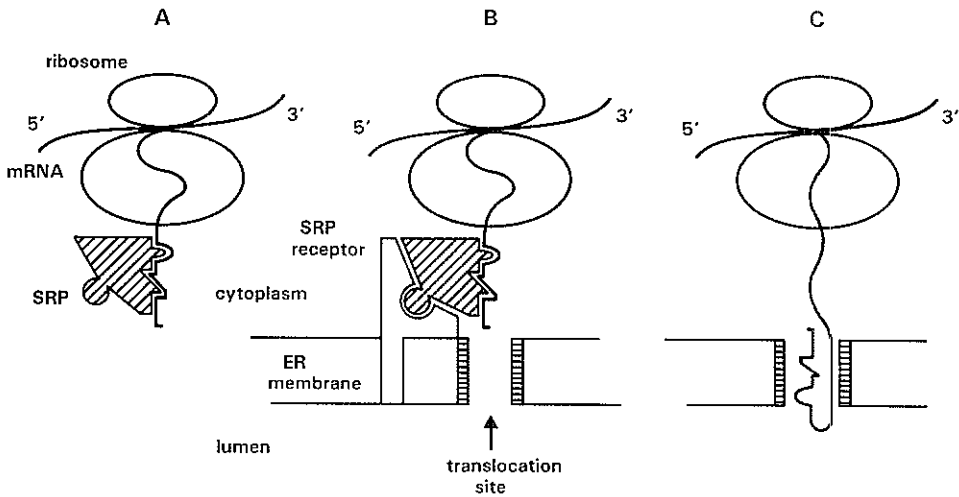






Figure 2. Protein translocation across the endoplasmic reticulum membrane



A) The signal peptide is recognized and bound by the signal recognition particle (SRP)

B) Targeting of the SRP-polypeptide-ribosome complex to the ER membrane

C) Translocation of the nascent polypeptide across the membrane

Figure 1. Flow diagram of protein traffic. The signals that direct protein movement are contained in the amino acid sequence. At each intermediate station a decision is made whether to stay (retention), or to travel further (anterograde transport). Endoplasmic reticulum (ER) resident proteins can be retrieved from the cis Golgi network by retrograde vesicular transport. The arrows with a question mark point to the possibility that ER resident proteins are also retrieved from later Golgi compartments although there is at present no direct evidence for these pathways. ( , mannose 6-phosphate receptor;

 phosphorylated lysosomal enzyme;  plasma membrane protein;  secretory glycoprotein;

-  exogenous protein being endocytosed;  plasma membrane protein being endocytosed)
- 1) Insertion of proteins in the plasma membrane
 - 2) Secretion of proteins either constitutive or regulated
 - 3) Endocytosis

al., 1981; Walter and Blobel, 1981). This recognition occurs as soon as the signal sequence extends from the ribosome (fig. 2A). The binding causes a transient arrest of translation. The SRP-ribosome-nascent protein complex is then targeted to the ER membrane where it binds to an ER-specific protein, the SRP receptor or docking protein (fig. 2B; Meyer et al., 1982; Gilmore et al., 1982). The signal peptide interacts with the translocation site in the ER membrane (fig. 2C; Wiedmann et al., 1987), and the SRP is released as a consequence from the complex and can bind another ribosome-signal peptide complex in the cytoplasm. Upon the release of the SRP, the translation activity of the ribosome is restored. The nascent polypeptide is translocated across the ER membrane during its translation. Simon and Blobel (1991) have demonstrated by electrophysiological techniques the existence of a protein-conducting channel in the ER membrane that mediates translocation of nascent polypeptides. Attached ribosomes keep the channel in an open conformation.

1.2 Modifications occurring in the rough endoplasmic reticulum

The amino-terminal signal sequence of most lysosomal and secretory proteins is cleaved off by signal peptidase shortly after translocation, but some plasma membrane proteins retain their signal peptide which then serves as a membrane anchor (fig. 3A). There is another class of plasma membrane proteins which lose their signal peptide but are subsequently anchored to the membrane via a glycosyl-phosphatidylinositol (GPI). This GPI-group is attached *en bloc* to the carboxyl-terminal end of the protein through ethanolamine, at the same time that a carboxyl-terminal sequence, that serves as signal for GPI-anchoring, is removed (fig. 3B; Ferguson & Williams, 1988; Lisanti et al., 1988; Baily et al., 1989; Hayashi et al., 1989; Ali & Evans, 1990). This occurs in the endoplasmic reticulum. Gerber et al. (1992) have studied the amino acid composition of the carboxyl-terminal signal sequence instrumental for GPI-anchoring. There appears to be a rule for predicting the cleavage/GPI addition site just as there is a rule for amino-terminal signal peptide cleavage (Von Heijne 1986; 1988). Studies with polarized epithelial cells have revealed that glycosyl-phosphatidylinositol membrane anchors contain information for targeting to the apical cell surface (Lisanti et al., 1988, 1989, 1990, 1991).

Besides membrane anchoring via the amino-terminal signal peptide or GPI, a large group of proteins is integrated in the lipid bilayer forming the endoplasmic reticulum membrane. These transmembrane proteins are classified in four groups according to their positioning in the membrane (Wickner & Lodish, 1985; Singer, 1990; fig. 3A). Type I and II proteins span the lipid bilayer once. The amino-terminal

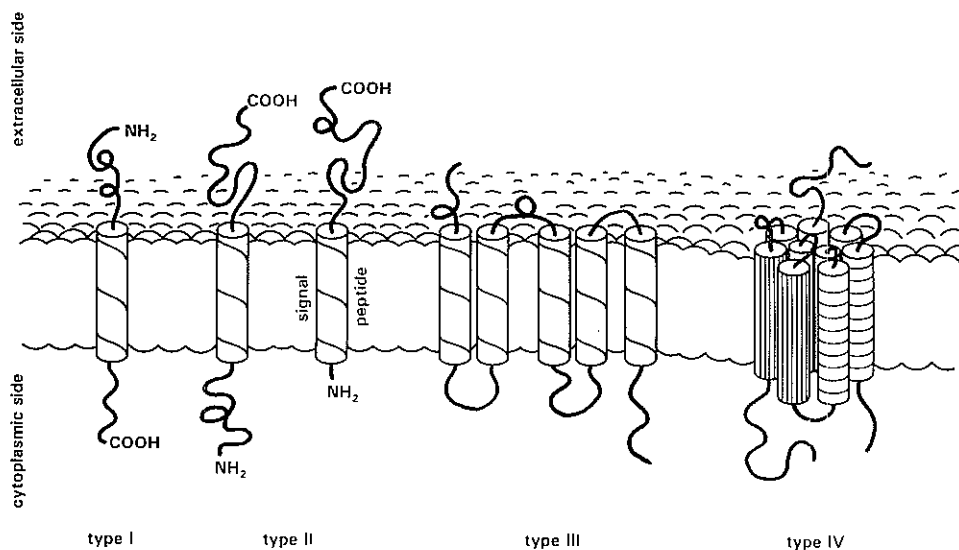
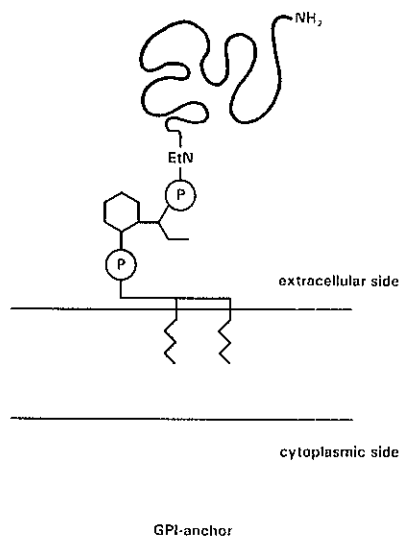


Figure 3A. Topology of different types of integral membrane proteins.

Figure 3B. Schematic diagram of a GPI-anchored protein. Membrane attachment is through phosphatidylinositol, which is linked to the carboxyl-terminus of the protein via a mannose-rich glycan and ethanolamine (EtN).



end of type I plasma membrane proteins is exposed to the extracellular space, whereas the amino-terminal end of type II glycoproteins is cytoplasmic. Type III transmembrane proteins contain multiple spanning domains whereby the amino-terminus can be situated on the extracellular or the cytoplasmic face of the membrane. Type IV transmembrane proteins are aggregates of multiple (identical) type III subunits.

Glycosylation is another important co-translational modification occurring in the rough endoplasmic reticulum (RER). An oligosaccharide structure composed of glucose, mannose and N-acetylglucosamine is transferred *en block* from a lipid carrier, dolichol-pyrophosphate, to the NH₂ group on the side chain of an asparagine residue of the protein, provided that the asparagine residue is in the conformation -Asn-X-Thr/Ser- (X being any amino acid, except proline). This N-linked Glc₃Man₉GlcNAc₂ oligosaccharide is the precursor of high-mannose, complex and hybrid type oligosaccharides. A number of oligosaccharide trimming reactions take place during subsequent transport of the polypeptide through the RER and the Golgi complex (reviewed by Elbein, 1984,1987; Fuhrmann et al., 1985), the first of which (removal of glucose) occurs in the RER.

Oligosaccharides can also be linked to the hydroxyl group on the side chain of a serine or threonine residue (O-linked oligosaccharides). In contrast to N-linked glycosylation, much less information is available about the assembly of O-linked sugar chains and about the intracellular location of the enzymes involved. O-linked sugar transfer in yeast starts in the endoplasmic reticulum by the transfer of a mannosyl residue from a dolichyl phosphate mannose intermediate to serine or threonine residues (Haselbeck & Tanner,1983; Strahl-Bolsinger & Tanner 1991). Additional mannosyl residues are connected. The initial glycosylation reaction in mammalian cells involves the transfer of GalNAc to serine or threonine residues and does not require a lipid intermediate. Galactose and sialic acid residues are subsequently attached. The subcellular site of the initial O-linked glycosylation reaction in mammalian cells is a point of discussion. Cell fractionation and immunocytochemical studies point to the Golgi complex (Hanover et al., 1982; Roth, 1984; Abeijon & Hirschberg, 1987) whereas studies with genetically modified proteins, that are trapped in the ER, suggest that the enzyme involved in the first O-linked sugar transfer reaction resides in the ER (Pathak et al., 1988; Kuwano et al., 1991).

Possible functions of protein glycosylation are:

- * proper functioning of the glycoprotein
- * participation in biological processes as cell-cell interactions; receptor-ligand interaction
- * induction of a specific three dimensional conformation
- * stability; resistance to proteases (especially O-linked sugars)

Other post-translational modifications in the RER involve folding of the polypeptide and formation of disulfide bridges (reviewed by Gething & Sambrook, 1992). It was thought that protein molecules fold spontaneously into their own functional conformation. Hydrophobic residues have a tendency to cluster, and become surrounded by more polar residues. Local interactions between peptide backbones give rise to α -helices and β -sheets. Proteins with binding sites for their own surface can assemble into dimers or oligomers. The hypothesis of protein self-assembly implicates that the aforementioned interactions are sufficient to produce a functional structure. Over the past years it became evident that in many cases interactions within and between proteins need to be controlled to reduce the formation of misfolded proteins. This control is generated by a group of proteins acting as chaperones (reviewed by Ellis & van der Vies, 1991). Molecular chaperones are defined by Ellis & Hemmingsen (1989) as proteins that mediate the correct assembly of other proteins but are not integrated in the final structure. Chaperones are thought to bind covalently to specific protein domains that are exposed only in the early stages of assembling, thereby forming a stable complex. This binding is reversible and requires sometimes ATP hydrolysis. Their way of action seems to be the inhibition of assembly modes that lead to nonfunctional protein structures. An example of a molecular chaperone in the endoplasmic reticulum of mammalian cells is binding protein BiP, previously described as immunoglobulin heavy chain binding protein), which is involved in folding and oligomerization of proteins (Munro & Pelham, 1986). It is demonstrated that Ca^{2+} levels modulate the association/dissociation of proteins with BiP (Lodish & Kong, 1990; Suzuki et al., 1991). Another molecular chaperone is protein disulphide isomerase (PDI), an enzyme which catalyses *in vitro* disulphide bond formation. It cleaves S-S bonds, thereby allowing proteins to search rapidly through different arrangements until the one with the lowest overall free energy is found. The same structure would finally be reached without PDI, but the process is accelerated in the presence of PDI (Edman et al., 1985; Freedman, 1989; Noiva & Lennarz, 1992). Proteins with peptidyl prolyl cis-trans isomerase (PPI-ase) activity belong also to the chaperone family. These proteins catalyse the isomerization of X-P peptide bonds (where X is any amino acid

and P is proline) and thereby accelerate the restructuring of proline containing polypeptides (Gething & Sambrook, 1992).

The capacity to sort proteins by conformational criteria provides the cell with a control system that prevents potential damage caused by the transport of defective gene products. The fate of misfolded proteins and incomplete complexes in the endoplasmic reticulum has been studied extensively over the past years. Lippincott-Schwartz et al. (1988) have studied the assembly of newly synthesized T cell receptor (TCR) subunits, which are composed of at least eight transmembrane chains encoded by six genes (Bonifacino et al., 1991). It became evident that unassembled or incompletely assembled TCR complexes were destroyed before they reached the Golgi complex by a process referred to as ER degradation (Klausner et al., 1990; Klausner & Sitia, 1990). Structural information for rapid degradation was demonstrated to reside in charged amino acid residues at defined positions within the transmembrane domain of the TCR α -chain (Bonifacino et al., 1990, 1991). The use of a permeabilized cell system, in which traffic between organelles is disrupted, revealed that the machinery responsible for degradation is present in the endoplasmic reticulum itself (Stafford & Bonifacino, 1991).

1.3 Transport routes between the endoplasmic reticulum and the Golgi complex

Having acquired the correct tertiary and quaternary structure newly synthesized proteins leave the endoplasmic reticulum. Transport of proteins between the rough endoplasmic reticulum and the Golgi complex is thought to be mediated by smooth vesicles that shuttle between specific, ribosome free, domains of the ER called transitional elements and the cis Golgi cisternae (Tartakoff, 1980; Farquhar, 1985; Pfeffer & Rothman, 1987). Temperature-sensitive yeast mutants that are defective in various stages of the secretory pathway have allowed the identification of several genes whose products function in ER to Golgi transport (reviewed by Hicke & Schekman, 1990; Newman & Ferro-Novick, 1990). It is more difficult to identify proteins that function as part of the ER to Golgi pathway of mammalian cells, as these cells are not suitable for the isolation of secretion mutants. Therefore, studies were performed on the routing of newly synthesized viral proteins in these cells. Immunoelectron microscopic studies of transport of newly synthesized viral membrane proteins at reduced (15°C) temperature revealed the accumulation of the proteins in tubulo-vesicular elements located between the ER and the Golgi complex (Saraste & Kuismanen, 1984). Also biochemical approaches like sucrose-D₂O gradients brought

evidence for the existence of an "intermediate compartment" between the ER and the Golgi complex (Lodish et al., 1987). The molecular dissection of vesicular transport from the ER, through the intermediate compartment, to the cis Golgi compartment was studied in semi-intact cells (Beckers et al., 1987, 1990; Plutner et al., 1991). The intermediate compartment is also named "salvage compartment" and was recently renamed to cis Golgi network (CGN).

Assembled proteins leave the ER by "default" (Wieland et al., 1987; Rothman 1987). This implies that the ER resident proteins must contain specific signals to be retained. The same holds for resident Golgi proteins and proteins which function in more distal compartments of the glycoprotein transport pathway. Thus, at consecutive transport stations proteins need to be sorted. Retention signals for soluble and type I transmembrane resident ER proteins have been identified. Soluble ER proteins like the aforementioned BiP and PDI and the glucose regulated protein, grp 94, all contain a C-terminal tetrapeptide sequence Lys-Asp-Glu-Leu (KDEL; Munro & Pelham 1987; Pelham et al., 1988; Pelham 1989a,b). Transplantation of the KDEL motif to the C-terminus of lysozyme (a protein that is normally secreted), causes retention of the modified lysozyme in the ER. Additional features of protein structure can be important to generate absolute ER retention, because adding the KDEL sequence to two other secretory proteins (rat growth hormone and the α -subunit of human chorionic gonadotropin) showed retardation of transport, but not retention in the ER compartment (Zagouras & Rose, 1989). There are indications, however, that the artificial addition of a retention signal to a protein can lead to differences in the presentation of the sorting signal (Johansen et al., 1990). Minor variations of the KDEL motif are allowed without loss of ER retention of proteins in mammals, plants and yeast. These are listed in Pelham (1989b, 1990), Haugejorden et al. (1991) and Denecke et al. (1992).

The mechanism of retention has been elucidated partially. It seems that proteins containing the KDEL or a related retention motif leave the ER by default, but are retrieved from the cis Golgi network (CGN) or from the Golgi complex and return to the ER (fig. 1). This implies a retrograde vesicle flow from the Golgi complex to the ER, and the existence of receptors that cycle between these two compartments. The receptors must have the property of binding to the retention signal in the CGN or the Golgi complex, and of release in the ER. Evidence that such a selective retrieval pathway exists came from studies in which the carbohydrate modifications that occur to proteins during transport were monitored (Pelham, 1988; Dean & Pelham, 1990; Peter et al., 1992). Experiments with the drug brefeldin A have also provided evidence for recycling of proteins from Golgi complex to ER (Lippincott-Schwartz et

al., 1990). The retrograde transport of proteins to the ER is microtubule dependent (Lippincott-Schwartz et al., 1990), whereas it is assumed that anterograde protein transport between ER and Golgi complex does not involve microtubules (Stults et al., 1989). The identification of yeast and mammalian "salvage receptors" has lent substance to this model (Lewis & Pelham, 1990; Lewis et al., 1990; Semenza et al., 1990; Vaux et al., 1990; Warren, 1990; Vaux & Fuller, 1991; Lewis & Pelham, 1992 a,b). Thus, proteins carrying retention signals are transported together with secretory proteins from the ER to the Golgi complex, they then return to the ER whereas the secretory proteins continue their way through the Golgi complex (Lewis & Pelham, 1992).

ER resident type I transmembrane proteins (fig. 3A) are also retained in the ER. Their retention motifs are laying in the cytoplasmic tails. Comparison of C-terminal amino acid residues and mutation analysis of the cytoplasmic C-terminal domain of different type I transmembrane ER resident proteins revealed four consensus motifs for retention (Pääbo et al., 1987; Nilsson et al., 1989; Jackson et al., 1990; Shin et al., 1991). These C-terminal motifs (-KKXX, -KXXXX, -RKXX, or -RXXKXX) function only in the presence of a transmembrane region. Studies on the retention signal of the E3/19K protein of adenovirus type 2 showed in addition the importance of neighbouring sequences of the -KK- motif (Gabathuler & Kvist, 1990). Not much is known about the mechanism by which transmembrane proteins are retained in the ER. An interaction with microtubules seems to be crucial for the retention of the E3/19K protein (Dahllöf et al., 1991). At present it is not known whether other ER resident transmembrane proteins are also capable to bind to microtubules.

1.4 Modifications occurring during transport through the Golgi complex

The Golgi complex is a polarized structure composed of sets of membrane bound smooth-surfaced cisternae surrounded by small vesicles (see for recent review Mellman & Simons, 1992). The Golgi complex has two distinct faces, a cis-face, where proteins enter the Golgi complex, and a trans-face, where proteins exit for multiple destinations. Histochemical studies have shown that the Golgi complex consists of three different compartments named cis-, medial-, and trans Golgi (Farquhar, 1985; Dunphy & Rothman, 1985; Griffiths & Simons, 1986). Transport of proteins from one cisternae to the next is mediated by non-clathrin coated vesicles (Farquhar & Palade, 1981; Strous et al., 1983; Saraste & Hedman, 1983). Elements of

the coat have recently been purified (Waters et al., 1991; Duden et al., 1991). A 110 kDa membrane protein associated with the cytoplasmic face of Golgi membranes, named β -COP, is the major coat component. The molecular basis of Golgi vesicular transport has been investigated by reconstitution experiments (reviewed by Rothman & Orci 1992).

Proteins are modified along their passage through the Golgi complex. Most common are alterations of the asparagine-linked oligosaccharides, O-linked glycosylation, specific proteolysis, sulfate addition, synthesis of glycosaminoglycans and fatty acid addition. The enzymes involved in oligosaccharide trimming work in concert along the different compartments. It is self-evident that proteins must have special structural features which serve as determinant for processing enzymes.

Three different types of N-linked sugar chains can be distinguished, namely high-mannose oligosaccharides which contain only mannose and N-acetylglucosamine residues, complex oligosaccharides which have in addition a variable number of galactose, sialic acid and sometimes fucose residues, and, hybrid oligosaccharides containing both complex and high-mannose peripheral branches.

Soluble proteins *en route* to the lysosomes acquire a lysosomal targeting signal in the Golgi complex by two reactions. The enzyme N-acetylglucosamine phosphotransferase transfers N-acetylglucosamine 1-phosphate from UDP-N-acetylglucosamine to specific mannose residues on high mannose oligosaccharide chains (Reitman & Kornfeld, 1981). Lazzarino & Gabel (1988) have demonstrated that this initial reaction occurs at a pre-Golgi site but a second N-acetylglucosamine 1-phosphate group can be added in the cis-Golgi compartment. The second reaction involves the enzyme N-acetylglucosamine-1-phosphodiester N-acetylglucosaminidase, localized in the medial Golgi cisternae, which removes the N-acetylglucosamine residue, whereby mannose 6-phosphate (M6P) is exposed as lysosomal targeting signal (Waheed et al., 1981). Baranski et al.(1990) have studied which structural features of lysosomal enzymes could possibly be instrumental for recognition by the phosphotransferase enzyme. It is unlikely that a signal is formed by a linear amino acid sequence since the lysosomal enzymes that have been cloned do not share significant sequence identity. Studies with chimeric proteins have shown that a signal can be formed by a three dimensional conformation involving residues in different loops of a lysosomal protein. Further characterization of the recognition domain of the lysosomal enzyme cathepsin D has revealed a surface patch that contains multiple interaction sites, with a strict positional requirement for a lysine residue (Baranski et al., 1991). Cathepsin D is a lysosomal aspartyl protease with two lobes, each of which contains one N-linked oligosaccharide chain. The most recent information is that both

lobes contain a recognition domain for N-acetylglucosamine phosphotransferase. Each domain effectuates with preference the phosphorylation of the carbohydrate chain in the same lobe, but the oligosaccharide in the opposite lobe can be phosphorylated as well (Baranski et al., 1992; Cantor and Kornfeld, 1992a). Flexibility of carbohydrate phosphorylation was also demonstrated for arylsulfatase A and Cathepsin D by introduction and deletion of carbohydrate side chains (Gieselmann et al., 1992; Cantor and Kornfeld, 1992b).

In one particular inheritable disorder named I-cell disease the first enzyme necessary for addition of this M6P marker is deficient. As a result lysosomal enzymes are secreted and the lysosomes are devoid of enzyme (Kornfeld, 1986).

The first indication that retention signals for Golgi resident proteins are different from those described for ER resident proteins, came from expression studies with corona virus glycoproteins. The infectious bronchitis virus (IBV) E1 glycoprotein is retained in the cis Golgi compartment when its cDNA is expressed in eukaryotic cells (Machamer et al., 1990). This E1 glycoprotein is a type III transmembrane protein (with the amino-terminus extending in the lumen and the carboxyl-terminus in the cytoplasm). The protein has three transmembrane domains, the first of which is required for cis Golgi retention (Machamer & Rose, 1987; Machamer et al., 1990; Swift & Machamer, 1991). For the E1 glycoprotein uncharged polar residues (Asn, Thr and Gln) that line one face of the predicted α -helix structure of the first transmembrane domain, seems to be important for retention (Swift & Machamer, 1991; Machamer, 1991).

The identification of genes encoding resident trans Golgi enzymes involved in carbohydrate modifications has revealed that all these genes encode transmembrane proteins with a type II orientation (fig. 3A; Paulson & Colley, 1989). The essential information for trans Golgi retention resides therefore most probably in the amino-terminal portion of the proteins (Colley et al., 1989). Analysis of genetically modified resident trans Golgi proteins has shown that the transmembrane domain together with flanking sequences, either on the cytoplasmic site alone or on both the luminal and the cytoplasmic site, contains targeting information (Nilsson et al., 1991; Munro, 1991; Tang et al., 1992; Teasdale et al., 1992; Wong et al., 1992; Colley et al., 1992; Russo et al., 1992). The sequence motif (Ser/Thr)-X-(Glu/Gln)-(Arg/Lys) occurs near a hydrophobic domain close to the amino-terminus of four glycosyltransferases and might be involved in Golgi retention (Bendiak, 1990). For β -1,4-galactosyltransferase it was shown that a cysteine and histidine residue located at the cytoplasmic side of the transmembrane domain are critical for Golgi retention (Aoki et al., 1992).

The mechanism of Golgi retention is unknown. Two theoretical models, a receptor-mediated model and an aggregation model, have been hypothesized by Machamer (1991).

1.5 Protein sorting in the trans Golgi network

The non-resident proteins that travel through the Golgi complex are classified into three groups according to their exit pathway (Griffiths & Simons, 1986):

- * plasma membrane proteins and constitutively secreted proteins
- * secretory proteins that are packaged into secretory granules
- * lysosomal enzymes

Secretory proteins that are transported directly to the cell surface without being stored in cytoplasmic granules follow the constitutive secretory pathway. Proteins that are stored in special granules which are released after an external stimulus are secreted along the regulated secretory pathway.

The place where sorting of proteins destined for different post Golgi compartments occurs is named trans Golgi network (TGN; Griffiths & Simons, 1986; Orci et al., 1987), also termed trans Golgi reticulum (TGR) or trans tubular network. Morphological studies have shown that the TGN exists of an extensive tubular reticulum on the trans side of the Golgi complex. Biochemical studies have revealed that proteins are sialylated in this compartment (Roth et al., 1985).

Newly synthesized plasma membrane proteins gather in smooth vesicles that pinch off from the TGN. Proteins destined for export via secretory granules concentrate in "vesicles" that have patches of clathrin on their cytoplasmic surface (Griffiths & Simons, 1986). From studies with fusion proteins it became clear that "sorting signals" are involved in the regulated pathway, while the constitutive pathway is based on default (Moore & Kelly, 1986). Regulated secretory proteins have a tendency to aggregate in the TGN. This is the result of a pH decrease and a calcium level increase in the TGN. Aggregation seems to be crucial for the segregation of regulated secretory proteins from "constitutive secretory proteins" (Chanat & Huttner, 1991). Both, regulated as well as constitutive secretory transport routes can be polarized in certain cell types (Kelly, 1985). Lysosomal enzymes equipped with the M6P marker cluster in coated vesicles which bud off from the TGN (Griffiths & Simons, 1986).

It has been reported that mature T cells have a control mechanism whereby aberrant molecules are retained in a "late cellular compartment". These molecules are

terminally sialylated, pointing to the TGN compartment. This quality control mechanism is developmentally regulated, because immature T cells lack the mechanism (Zamoyska & Parnes, 1988).

1.6 Lysosomal protein targeting

At present three different pathways for lysosomal enzyme targeting are known. Soluble lysosomal enzymes follow a pathway that involves a membrane receptor. In 1981, Sahagian et al., reported the isolation of an integral membrane protein with an apparent molecular mass of 275 kDa that is capable of binding to the M6P marker on lysosomal enzymes, and therefore is named mannose 6-phosphate receptor (MPR). This receptor can also bind IGF-II. Some murine tissue culture cell lines which are receptor deficient, are still able to sort 30-40% of their newly synthesized lysosomal enzymes to lysosomes, indicating the presence of a targeting mechanism that is independent of the 275 kDa MPR (Gabel et al., 1983). Hoflack & Kornfeld (1985) have demonstrated the presence of a second MPR with a molecular mass of approximately 46 kDa in these murine cell lines, that also participates in the delivery of acid hydrolases to lysosomes. The large receptor binds ligands independent of divalent cations (cation independent; CI-MPR), whereas the small receptor exhibits enhanced ligand binding affinity in the presence of divalent cations (cation dependent; CD-MPR) (Hoflack & Kornfeld, 1985; Hoflack et al., 1987). The cloning of the cDNAs coding for both receptors has provided insights into their structures and has revealed that the two receptors are derived from a common ancestor gene (reviewed by Kornfeld & Mellman, 1989; Kornfeld, 1990). The subcellular distribution of both receptors is almost identical (Bleekemolen et al., 1988). The MPRs are present in the Golgi complex, with the highest concentration in the trans Golgi network (Geuze et al., 1985; Willemsen et al., 1988). MPRs are also present at the plasma membrane and in the endosomal compartment.

Newly synthesized lysosomal proteins equipped with the M6P marker bind to the MPR in the trans Golgi network whereafter the receptor-ligand complex clusters in clathrin coated vesicles which pinch off from the TGN (fig. 1 and 4; Campbell et al., 1983; Geuze et al., 1985; Lemansky et al., 1987). The coated vesicles fuse with a late endosomal acidic compartment. Due to the low pH in this compartment, receptor and ligand dissociate (Geuze et al., 1985, 1988; Brown et al., 1986; Griffiths et al., 1988). The MPR receptors then cycle back to the Golgi complex and/or the plasmamembrane (Duncan & Kornfeld, 1988). The uncoupled lysosomal enzymes are transported to lysosomes.

A minor part of newly synthesized lysosomal proteins is secreted (Von Figura & Hasilik, 1988; Kornfeld, 1986; 1987). Secreted lysosomal proteins carrying the M6P marker are able to bind to the CI-MPR exposed on the plasmamembrane. They are internalized via a process that is named receptor-mediated endocytosis (fig. 1; reviewed by Smythe & Warren, 1991). The CD-MPR is not involved in this process, probably due to an inability to bind ligands at the cell surface (Stein et al., 1987).

Clustering of ligand-MPR complexes in clathrin coated pits at either the TGN or the plasmamembrane depends on an interaction of the receptor with adaptor proteins. Adaptors form an inner layer of the coat, between the outer clathrin lattice and the membrane. Adaptor proteins are present as a complex, HA-1/AP1 (in the TGN) or HA-2/AP2 (at the plasmamembrane). The complex exists of a number of components, including the α -, β - and γ -adaptins (for recent review see Pearse & Robinson, 1990; Duden et al., 1991). Pearse (1988) demonstrated that the HA-2/AP2 adaptor complex at the plasmamembrane is able to bind to the cytoplasmic tail of the CI-MPR. This association is dependent on the presence of tyrosine residues in the cytoplasmic domain of the CI-MPR (Lobel et al., 1989; Glickman et al., 1989; Ktistakis et al., 1990; Canfield et al., 1991). Tyrosine residues have been identified in the cytoplasmic domains of a number of proteins that are rapidly internalized and they appear to be a crucial element of the endocytosis signal. The tyrosine residues involved, can be substituted with phenylalanines, indicating the requirement of an aromatic residue rather than a tyrosine specifically (Canfield et al., 1991). A large hydrophobic residue (Val, Phe, Ile), three positions away from the tyrosine residue, was shown to be another important element for rapid internalization (Jadot et al., 1992). The HA-1/AP1 adaptor complex binds to the cytoplasmic tail of the CI-MPR in the TGN, but this interaction is not based on tyrosine residues only (Glickman et al., 1989). Recent studies of Johnson and Kornfeld (1992a/b) mention the structural motifs instrumental in Golgi sorting of lysosomal enzymes. The CI-MPR contains two signals for efficient lysosomal enzyme sorting in the TGN. The tyrosine containing sequence in the carboxyl terminus of the receptor, which also functions as internalization signal, and a second sorting motif which is formed by a carboxyl terminal peptide sequence Leu-Leu-His-Val (Johnson and Kornfeld 1992b). Impaired sorting occurs if one of the signals is altered, while complete loss of MPR function is only observed when both sorting signals are altered. A His-Leu-Leu sequence near the carboxyl terminus of the CD-MPR was shown to be instrumental in the sorting of lysosomal enzymes in the TGN (Johnson and Kornfeld 1992a). Both, the CD- and CI-MPR, contain a Leu-Leu motif for sorting of lysosomal enzymes in the Golgi complex.

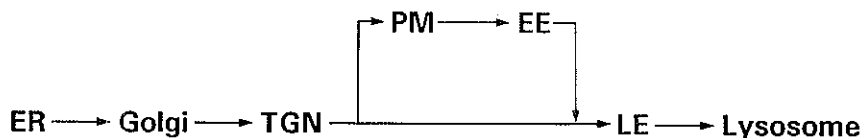


Figure 4. Direct and indirect routing of proteins to the lysosome. ER, endoplasmic reticulum; TGN, trans Golgi network; PM, plasma membrane; EE, early endosome; LE, late endosome.

The first indication that mannose 6-phosphate receptor independent lysosomal transport pathways exist was the observation that not all lysosomal enzymes are secreted by I-cell fibroblasts which cells are deficient in synthesis of the M6P recognition marker. Enzymes that are membrane-associated (glucocerebrosidase) or those that are synthesized as integral membrane protein precursors (acid phosphatase) are retained in I-cell fibroblasts and transported to the lysosomes (Neufeld & McKusick, 1983; Van Dongen et al., 1984). Moreover, both glucocerebrosidase (Aerts et al., 1986, 1988; Rijnbout et al., 1991) and acid phosphatase (Waheed et al., 1988) do not acquire phosphorylated mannose residues even when synthesized in normal fibroblasts. Also the high residual activity of acid α -glucosidase in I-cell disease may be due to membrane association and MPR-independent transport (Tsuji and Suzuki, 1987; Hoefsloot et al., 1990; Wisselaar et al., 1993).

MPR-independent lysosomal enzyme transport pathways may differ per cell type, as was shown for acid phosphatase. Acid phosphatase is synthesized as an integral membrane protein with a short carboxyl-terminal tail of 18 amino acids at the cytosolic side. In fibroblasts, intestinal epithelial cells and in transfected baby hamster kidney cells (BHK) acid phosphatase has been demonstrated immunocytochemically in lysosomes, but also at the cell surface (Parenti et al., 1987; Braun et al., 1989; Willemsen et al., 1991). In transfected BHK cells, acid phosphatase is transported from the trans Golgi network to the cell surface, whereafter rapid internalization takes place via endocytosis (Fig. 4; Braun et al., 1989). It was demonstrated by mutation analysis that internalization of the mannose 6-phosphate receptor is dependent on the presence of a tyrosine residue in the cytoplasmic domain (Peters et al., 1990). This tyrosine residue occurs in the structural context of a tight β -turn

(Eberle et al., 1991). Most of the internalized acid phosphatase molecules are transported back to the cell surface. It was shown that acid phosphatase molecules cycle approximately 15 times between the plasmamembrane and the endosomes before they enter the lysosomes. In the lysosomes acid phosphatase is released from the membrane by proteolytic cleavage. The transmembrane and the cytosolic tail are clipped off (Waheed et al., 1988). Another transport pathway is observed in hepatocytes, where acid phosphatase transport does not involve passage of the plasmamembrane, instead, it is directly routed from TGN via late endosome to the lysosomes (Tanaka et al., 1990 a,b).

Proteins that share properties with lysosomal acid phosphatase are integral lysosomal membrane proteins. Their identification and characterization was generated by the production of antibodies against lysosomes. These antibodies have defined a group of antigens ranging in molecular mass from 100 kDa to 120 kDa (Marsh et al., 1987). During the past years, the cloning of cDNAs coding for the major glycoproteins of the lysosomal membrane have revealed two distinct but highly homologous groups named lgp-A and lgp-B (Granger et al., 1990). Sequence comparison suggests that differences within each group reflect species variations of the same protein and that lgp-A and lgp-B have diverged probably from a common ancestor. Both lgp-A and lgp-B are type I transmembrane proteins with large highly glycosylated luminal domains (for review see Kornfeld & Mellman, 1989). The oligosaccharides are not equipped with the M6P marker, pointing to MPR-independent routing (Krentler et al., 1986; Barriocanal et al., 1986; Howe et al., 1988). Studies on intracellular transport of lysosomal membrane proteins has yielded controversial results (Lippincott-Schwartz & Frambrough, 1987; Green et al., 1987; Kornfeld & Mellman, 1989; Harter & Mellman, 1992; Carlsson & Fukuda, 1992; Mathews et al., 1992). There are two possible routes for integral lysosomal membrane proteins to reach the lysosomes. The first is that lgp's are sorted in the TGN and transported via endosomes to lysosomes. The second is that lgp's are first transported to the cell surface and then selectively internalized and transported to the lysosomes.

Mutation analysis of human lgp-A in combination with expression studies has shown that a tyrosine residue in a specific position in the cytoplasmic domain is necessary for lysosomal targeting. This sorting signal may be recognized both in the TGN and at the plasmamembrane (Williams & Fukuda, 1990). Mutants in which the tyrosine residue is replaced accumulate at the cell surface. These mutant molecules are probably transported to the cell surface via the constitutive secretory pathway. Further, it was demonstrated that the cytoplasmic tail enables a reporter molecule to be delivered to the lysosomes (Williams & Fukuda, 1990). All presently known lgp-A

and lgp-B molecules of different animal species contain a tyrosine residue in their cytoplasmic domain (for recent review see Fukuda, 1991). The tyrosine residue in proper context is nowadays believed to be a general endocytosis signal.

The targeting of rat lysosomal membrane protein (LIMP II), which has no significant homology with lgp-A or lgp-B, does not require a tyrosine residue in the cytoplasmic tail (Vega et al., 1991 a,b). LIMP II has in contrast to lgp-A and lgp-B proteins an uncleaved signal peptide, and contains a second hydrophobic domain, indicating that LIMP II is a type III transmembrane protein which has two membrane spanning domains. Thus, at least one other sorting signal for lysosomal membrane glycoproteins must exist. Johnson and Kornfeld (1992b) suggest that a Leu-Ile sequence that is present in the carboxyl terminus of LIMP II could serve as sorting motif in analogy to the Leu-Leu sorting motif of MPR's.

1.7 Transport routes in polarized cells

Epithelial cells cover the surface of organisms, thereby forming a barrier between the organism and the environment. Epithelial cells have a polarized appearance. Their plasma membrane is divided by tight junctions in two distinct regions, the apical domain which faces the environment, and the basolateral domain, which attaches the cell to an extracellular matrix and maintains interaction with neighbouring epithelial cells via specialized intercellular junctions. Apical and basolateral domains have very different protein and lipid compositions. Lipid polarity in epithelia appears to be expressed only in the outer leaflet of the bilayer (van Meer & Simons, 1986). This lipid polarity is maintained by tight junctions.

The polarized distribution of membrane proteins and lipids in the plasmamembrane results from the ability of epithelial cells to sort newly synthesized molecules to their correct final destinations. Studies on protein sorting in various polarized cell types, like Madin Darby canine kidney (MDCK), hepatocytes or HepG2 cells and the enterocyte like colon carcinoma cell line Caco-2, have indicated that the routing of proteins can differ per cell type (recently reviewed by Mostov et al., 1992). Two pathways, a direct and an indirect, are distinguished in polarized cells. Newly synthesized proteins can travel directly from the trans Golgi network (TGN) to either the apical or the basolateral surface. Some proteins travel first to the basolateral surface and from there via endocytosis and transcytosis to the apical plasma membrane (indirect pathway). Studies in MDCK cells on the routing of viral envelope glycoproteins, budding from either the apical surface (influenza hemagglutinin) or from the basolateral surface (G-protein of vesicular stomatitis virus), have shown that

both viral proteins travel together to the TGN. In the TGN they are sorted in different vesicles that carry them to the apical and basolateral plasmamembrane, respectively (Rindler et al., 1984; Griffiths & Simons, 1986; Gottlieb et al., 1986). Also *de novo* synthesized endogenous proteins in MDCK cells are delivered directly from the TGN to their destination (Caplan et al., 1986; Le Bivic et al., 1990). In contrast, in hepatocytes, both the apical and basolateral plasmamembrane proteins are first transported to the basolateral plasmamembrane domain, whereafter the apical proteins are sorted, endocytosed and transported to the apical membrane domain (reviewed by Bartles & Hubbard, 1988).

It is clear that targeting to a specific plasmamembrane domain in polarized cells requires sorting signals. Sorting signals can direct a protein to a pathway or exclude it from other pathways. Expression studies of genetically modified proteins have been instructive in the identification of specific sorting signals. As already mentioned in chapter 1.2, a glycosyl-phosphatidylinositol (GPI) anchor is a signal for routing to the apical plasmamembrane domain (Lisanti et al., 1988; 1989; 1991; Lisanti & Rodriguez-Boulan, 1990). GPI anchors are connected to type I transmembrane proteins (Singer et al., 1990). Studies were performed in which the transmembrane anchor of a normally basolateral protein was replaced with a GPI anchor. The genetically modified protein was targeted to the apical surface instead of to the basolateral membrane. This experiment demonstrated that a GPI anchor is an apical sorting signal. The mechanism of GPI linked protein sorting may involve clustering with glycosphingolipids in the trans Golgi network (Lisanti & Rodriguez-Boulan, 1990; Simons & Wandinger-Ness, 1990). Apical plasmamembrane proteins linked via a GPI anchor are trehalase and alkaline phosphatase.

Apical targeting information for transmembrane proteins like aminopeptidase N (APN), sucrase-isomaltase, dipeptidyl-peptidase IV (DPP-IV) and viral glycoproteins is not carried in the cytoplasmic tail of these proteins and may be present in their luminal domains (McQueen et al., 1986; Roth et al., 1987; Compton et al., 1989; Vogel et al., 1992; Weisz et al., 1992). The route taken to the apical surface seems to be cell type specific. In hepatocytes, DPP-IV and APN use the transcytotic route (Bartles et al., 1987). In CaCo-2 cells, DPP-IV and APN use both the direct as well as the transcytotic route (Matter et al., 1990a; Klumperman et al., 1991). In transfected distal kidney tubule (MDCK-II) cells, DPP-IV and APN use a direct apical pathway, whereas in transfected proximal kidney tubule (LLC-PK₁) cells, DPP-IV uses both a direct and an indirect pathway (Wessels et al., 1990; Low et al., 1991a,b; Casanova et al., 1991b).

It is noteworthy that elements of the cytoskeleton, especially microtubules, facilitate transport of vesicles containing newly synthesized apical proteins but not of vesicles with basolateral proteins. A correlation between an intact microtubule network and direct or indirect apical delivery has been demonstrated (Rindler et al., 1987; Achler et al., 1989; Eilers et al., 1989; Matter et al., 1990). The microtubules in polarized epithelial cells are ordered with their plus ends near the basolateral surface and their minus ends near the apical surface. Translocation of apical transport vesicles to the apical plasmamembrane may be facilitated by microtubules, possibly via microtubule motors (Vale & Goldstein, 1990; Stebbings, 1990).

Until recently it was generally assumed that apical sorting was dependent on specific signals, whereas transport to the basolateral membrane would not require such signals, but would instead occur by default (Rodriguez-Boulant & Nelson, 1989; Simons & Wandinger-Ness, 1990). This view is changing, since evidence was obtained for the existence of basolateral sorting signals. For instance, the polymeric immunoglobulin receptor (pIgR) travels from the TGN to the basolateral plasmamembrane, where it binds IgA and IgM, and is then transcytosed to the apical surface (Mostov & Deitcher, 1986). It was demonstrated that a 14 amino acid segment in the cytoplasmic tail of pIgR directs this receptor to the basolateral domain (Casanova et al., 1991a). A mutant pIgR receptor lacking these 14 amino acids, is directly targeted from the TGN to the apical plasmamembrane domain. The evidence that the 14 amino acid segment is a signal for basolateral targeting came from an experiment in which this 14 amino acid sequence was added to alkaline phosphatase (an apical membrane protein usually anchored via GPI). The mutant protein was redirected to the basolateral membrane.

The cytoplasmic domains of other proteins also appeared to be important in basolateral membrane routing. This has been investigated using MDCK II cells, stably expressing the Fc receptor, rat IgG A or the LDL receptor. The targeting of newly synthesized integral lysosomal membrane proteins *en route* to the lysosomes includes passage of the basolateral plasmamembrane in transfected MDCK-II cells (Hunziker et al., 1991; Nabi et al., 1991). Mutations or deletions in the cytoplasmic domains of these proteins redirects the mutant proteins to the apical surface (Hunziker et al., 1991). Also the alternative approach was taken. For instance the introduction of a tyrosine residue at the position of the usual cysteine in the cytoplasmic tail of hemagglutinin (HA) changes the direction of sorting from apical to basolateral (Brewer et al., 1991; Le Bivic et al., 1991). These results contradict the model that basolateral targeting is a default mechanism. The identified basolateral sorting signals of the Fc receptor and rat IgG A are structurally closely related to the signal required for endocytosis via clathrin coated pits (Hunziker et al., 1991). It is suggested that at

least two distinct signals for basolateral transport exist, one dependent and one independent of a tyrosine residue. The first signal overlaps or is similar to that involved in endocytosis. Yokode et al. (1992) have suggested that the basolateral sorting motif of the LDL receptor and the polymeric immunoglobulin receptor is formed by four amino acids, spaced at intervals of three, possibly facing the same side of an α -helix.

The mechanism of basolateral sorting is purely speculative at present. The two basolateral sorting signals may specify transport along a common pathway, or may specify distinct pathways from the TGN to the basolateral plasmamembrane domain. The observed similarity between the basolateral sorting signal and signals involved in endocytosis, via clathrin coated pits, makes it likely that there will be some mechanistic relationship. It is possible that basolateral membrane proteins cluster in coated vesicles in the TGN, which directs them directly to the basolateral membrane or to endosomes from which they are subsequently transported to the basolateral plasmamembrane (Mostov et al., 1992). An alternative possibility is that non-clathrin coated vesicles are involved. Further analysis with cell free systems and permeabilized cells will provide additional information on TGN sorting (Bennett et al., 1988; Wandinger-Ness et al., 1990; Salamero et al., 1990; Gravotta et al., 1990).

At present one signal has been identified for transcytosis (Casanova et al., 1990). Phosphorylation of a serine residue in the cytoplasmic domain of the polymeric immunoglobulin receptor is required for efficient transcytosis. Mutations in the cytoplasmic domain replacing this serine residue with alanine, generated a receptor that is transcytosed slowly. Replacement of serine with aspartic acid (which mimics the negative charge) results in rapid transcytosis, indicating that the negative charge mediates this effect. The use of phosphorylation as a molecular switch enables the cell to regulate transcytosis in response to external stimuli (Casanova et al., 1990).

1.8 References

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Chapter 2

Acid α -glucosidase, sucrase-isomaltase and their deficiencies

2.1 Acid α -glucosidase and glycogenosis type II

Acid α -glucosidase (EC 3.2.1.3) is a glycoprotein with an acidic pH optimum that performs its function in the lysosomes. The function of acid α -glucosidase is to degrade glycogen that has entered the lysosomes by means of autophagy. Glycogen is a polymer of glucose and the main store of carbohydrate in animal cells. Glycogen is particularly abundant in liver and muscle. The branched structure of the polymer contains 20,000 to 30,000 glucose units. These glucose units are linked to each other by α -1,4 or α -1,6 (branching points) glycosidic linkages. Acid α -glucosidase hydrolysis both α -1,4 and α -1,6 linkages, allowing complete degradation of glycogen to glucose. Glucose is transported out of the lysosome and is reutilized in the cytoplasm. Other natural substrates of acid α -glucosidase are the disaccharides maltose (α -1,4 glycosidic linkage) and isomaltose (α -1,6 glycosidic linkage). Acid α -glucosidase activity is often measured with the artificial substrate 4-methylumbelliferyl- α -D-glucopyranoside.

The gene coding for human acid α -glucosidase consists of 20 exons and is located on chromosome 17q23→ter (Hoefsloot et al., 1990a; Solomon et al., 1979). The cDNA encodes a protein of 952 amino acids (Hoefsloot et al., 1988). In accordance, acid α -glucosidase is synthesized as a protein of approximately 100 kDa. During translocation across the endoplasmic reticulum membrane N-linked oligosaccharides are added to the protein, which enlarges the molecular mass with approximately 12 kDa (van der Horst et al., 1987). In pulse labelling experiments, the first detectable acid α -glucosidase species has a molecular mass of approximately 110 kDa (Hasilik & Neufeld, 1980a; Oude Elferink et al., 1984a,b; Reuser et al., 1985). *En route* through the Golgi complex, mannose residues of the oligosaccharide side chains are phosphorylated (Hasilik & Neufeld, 1980 b; Reuser et al., 1985). The acid α -glucosidase precursor of 110 kDa is proteolytically processed via a long lived 95 kDa intermediate form to mature species of 76 kDa and 70 kDa. The latter two species are the most abundant α -glucosidase forms in tissues and cultured cells of healthy individuals (Hasilik & Neufeld, 1980a,b; Martiniuk et al., 1984; Reuser et al., 1985; 1987).

Deficiency of acid α -glucosidase results in lysosomal glycogen accumulation, predominantly in skeletal and heart muscle cells. This lysosomal storage disorder was first described in 1932 by the Dutch pathologist Dr. J.C. Pompe and became known as

Pompe's disease. Later, it was classified as glycogen storage disease type II (GSD II or glycogenosis type II). The clinical expression of GSD II is variable. In the most severe form of the disease symptoms are present at birth or they appear shortly after. Characteristic features of this infantile form of GSD II are cardiomegaly, hepatomegaly, and severe hypotonia. The rapidly progressive dysfunction of the organs involved causes death in the first or second year of life. Symptoms may also develop gradually during the first decade of life, and become fatal before the third decade. This clinical pattern is called late infantile or juvenile GSD II. Other patients may be free of symptoms until their second or third decade of life. This adult form of GSD II is generally less progressive but skeletal muscle weakness affecting the respiratory muscle is a life threatening problem.

The clinical expression of the disease is correlated with the extent of acid α -glucosidase deficiency. In the infantile cases, the acid α -glucosidase activity is close to zero, whereas juvenile and adult patients show often a significant acid α -glucosidase activity. Up to 25% of the normal activity has been measured in adult patients (Mehler & DiMauro, 1977; Shanske et al., 1986; Reuser et al., 1978, 1987; van der Ploeg et al., 1987, 1988, 1989; Hoefsloot et al., 1990b), but some adult patients have been reported with an exceptionally low residual activity (Beratis et al., 1983; Reuser et al., 1987; Martiniuk et al., 1991; Hermans et al., 1993). Effective treatment for glycogenosis type II is currently not available.

Lysosomal α -glucosidase deficiency and vacuolar deposition of glycogen has also been observed in animal species. Glycogenosis type II has been identified in Brahman and Shorthorn cattle (O'Sullivan et al., 1981; Richards et al., 1977), Lapland dog (Mostafa et al., 1970; Walvoort et al., 1982), cat (Sandström et al., 1969), Corriedale sheep (Manktelow & Hartley, 1975) and in Japanese quail (Murahami et al., 1980).

In recent years a number of compounds have been shown to induce lysosomal glycogen storage. Acarbose, castanospermine, and nojirimycin and its derivatives are inhibitors of α -glucosidase. Intraperitoneal injection of these compounds in rats induces intralysosomal glycogen storage in liver and other organs (Lüllmann-Rauch, 1982; Chambers et al., 1982; Saul et al., 1985). These model systems are useful for studying the pathogenesis of glycogenosis type II.

A quite different application of these α -glucosidase inhibitors is their use in treatment of diabetes mellitus (Sachse & Willms, 1979; Taylor et al., 1986; Arends & Willms, 1986; Katsilambros et al., 1986; Holt et al., 1988; Kingma et al., 1992). The drugs inhibit intestinal sucrase and maltase activity thereby preventing a rapid rise of

the blood glucose level after ingestion of starch, sucrose or maltose (Lembcke et al., 1985; Samulitis et al., 1987). The nojirimycin derivatives are more potent inhibitors than acarbose, but they are absorbed in the intestine with the potential risk of disturbing cellular metabolism during long periods of treatment (Arends & Willms, 1986). In contrast, acarbose is not absorbed in the intestine, but causes gastrointestinal side effects (Kingma et al., 1992).

2.2 Sucrase-isomaltase and congenital sucrase-isomaltase deficiency (CSID)

The disaccharidase sucrase-isomaltase (EC 3.2.1.48 & EC 3.2.1.10) is a glycoprotein that is active at neutral pH, and performs its function at the apical plasma membrane of the small intestinal epithelial cells. Both subunits split maltose (α -1,4 linkage). In addition, the sucrase subunit splits sucrose (α -1,2 linkage) and turanose (α -1,3 linkage), and the isomaltase subunit splits the α -1,6 glycosidic linkage in isomaltose and in a number of branched α -dextrins.

The sucrase-isomaltase complex is a heterodimer composed of two similar but not identical subunits. The complex is synthesized as a single polypeptide of approximately 185 kDa in humans (Naim et al., 1988b) or 203 kDa in rabbits (Hunziker et al., 1986). The amino terminus of the isomaltase subunit contains a number of positive charged amino acid residues, followed by a hydrophobic stretch of approximately 20 amino acid residues, an ideal conformation for a signal peptide. Upon translocation across the endoplasmic reticulum membrane N-linked high mannose type of oligosaccharides are attached to the polypeptide precursor. The precursor is not released from the membrane by signal peptidase, and is therefore a type II transmembrane protein. Thus, the signal peptide of sucrase-isomaltase has a dual function. The precursor is not enzymatically active (Sjöström et al., 1985). During intracellular transport through the endoplasmic reticulum and the Golgi cisternae, N-linked high mannose oligosaccharides are trimmed and converted to complex type of sugar chains, and O-linked sugars are attached. The precursor is enzymatically active after these sugar chain modifications (Hauri et al., 1979). The sugar chains enlarge the apparent molecular mass of sucrase-isomaltase in both human and rabbit with approximately 60 kDa (Hunziker et al., 1986; Naim et al., 1988b). From the trans Golgi network the sucrase-isomaltase precursor is transported to the apical plasma membrane domain. Upon exposure in the intestinal lumen pancreatic proteases cleave the precursor into its subunits (Hauri et al., 1980, 1982; Montgomery et al., 1981; Sjöström et al., 1980, 1982; Danielsen, 1982). The sucrase subunit remains associated with the isomaltase subunit by non-covalent interactions (Frank et al., 1978; Brunner et al., 1979; Hunziker et al., 1986).

Hereditary sucrose malabsorption was first described by Weijers et al. (1960). Shortly thereafter it became clear that isomaltose absorption was also deficient in this condition. The clinical manifestations of congenital sucrase-isomaltase deficiency (CSID) are watery osmotic-fermentative diarrhoea, which may lead to dehydration and malnutrition. Failure to thrive and other symptoms are severe in the young child, but there is a tendency toward spontaneous improvement of symptoms with age. Patients are usually treated during the first three years of life and this treatment consists of elimination of sucrose, glucose polymers and starch from the diet. In adult life, excessive amounts of starch should be avoided.

Sucrose-isomaltose malabsorption is a heterogeneous condition: all patients lack sucrase activity, some patients have residual isomaltase activity, whereas others lack isomaltase activity completely. At present five different types of molecular defect are distinguished (Hauri et al., 1985; Naim et al., 1988a; Fransen et al., 1991). Type I is characterized by intracellular accumulation of the high mannose sucrase-isomaltase precursor, possibly in the endoplasmic reticulum. In type IV most high mannose precursor molecules are trapped in the endoplasmic reticulum, whereas the few that escape are missorted to the basolateral membrane. Accumulation in the Golgi complex and degradation prior to complex glycosylation is the second type of defect. Catalytically altered enzyme present at the apical plasma membrane is characteristic for a type III defect. Type V represents the condition in which sucrase-isomaltase is split intracellularly, whereafter the isomaltase subunit is normally transported to the apical plasma membrane, while the sucrase subunit is degraded intracellularly.

2.3 Similarities between α -glucosidase and sucrase-isomaltase

Based on the cDNA and the amino acid sequence of human lysosomal α -glucosidase and mammalian sucrase-isomaltase it has to be concluded that the genes coding for these enzymes have evolved from the same ancestral gene whereby the sucrase-isomaltase gene was generated by a duplication (Hunziker et al., 1986; Hoefsloot et al., 1988). An α -glucosidase and a glucoamylase with a high degree of homology to lysosomal α -glucosidase and sucrase-isomaltase occur in *Candida tsukubaensis* and *Schwanniomyces occidentalis*, respectively (Kinsella et al., 1991; Naim et al., 1991; Chantret et al., 1992). When the primary structure of these glucohydrolases are compared the conservation of the catalytic site domain is most prominent. A conserved aspartic acid in rabbit sucrase and isomaltase was demonstrated to be the essential carboxylate (Quaroni & Semenza, 1976). It was demonstrated recently that also the aspartic acid residue present in the homologous

domain of acid α -glucosidase is critical for catalytic function (Hermans et al., 1991).

The most significant sequence differences are located in the amino terminal domains. These variations could account for the preferred localization of the various enzymes, being lysosomal (lysosomal acid α -glucosidase), at the plasma membrane (sucrase-isomaltase) or extracellular (Sch. occidentalis glucoamylase, C. tsukubaensis α -glucosidase).

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Chapter 3

Results and discussion

The experimental work that has led to this thesis is described in detail in chapters 4-8. In chapter 4 and 6 the emphasis is on the structural aspects of acid α -glucosidase, in particular (i) the function of the signal peptide and its role in intracellular routing, (ii) the proteolytic processing of acid α -glucosidase and its consequences for enzyme function, (iii) the glycosylation of acid α -glucosidase and its role in enzyme stabilization and transport. The effect of an α -glucosidase inhibitor on the proteolytic processing and the catalytic function of acid α -glucosidase is described in chapter 5. Chapter 7 deals with the routing of acid α -glucosidase in polarized cells, whereby special attention is given to the homology of acid α -glucosidase and the intestinal brush border enzyme complex of sucrase-isomaltase. The biosynthesis of the sucrase-isomaltase enzyme complex is described in detail in chapter 8.

An overview of the experimental work is given in the following paragraphs. Each paragraph covers a different aspect, and the results are discussed in broader perspective.

3.1 The signal peptide of acid α -glucosidase and sucrase-isomaltase

Both lysosomal α -glucosidase and the brush border enzyme complex sucrase-isomaltase are glycoproteins and their synthesis and organelle specific targeting requires co-translational import in the endoplasmic reticulum. Both proteins are, therefore, equipped with a signal peptide at their amino-terminal ends. Characteristic features of signal peptides are found in both sequences (Hoefsloot et al., 1988; Hunziker et al., 1986; chapter 4). The n-region of the signal peptide of acid α -glucosidase and sucrase-isomaltase has a positive net charge. The adjacent h-region is typically rich in apolar residues. Leucine is the most predominant residue in the h-region of α -glucosidase whereas, the comparable region of sucrase-isomaltase is rich in iso-leucine. The difference might be of functional significance since iso-leucine and valine residues are frequently found in uncleaved signal peptides, and leucine and alanine in proteins with a cleavable signal peptide (Von Heijne 1986; 1988; Von Heijne & Gavel, 1988). The secondary structure as predicted by the Garnier, Osguthorpe and Robson analysis (1978) of the h-region of α -glucosidase and sucrase-isomaltase is that of an α -helix, an ideal conformation to span the membrane. Potential signal peptidase cleavage sites are located in the luminal c-region. The

predicted signal peptide cleavage site of α -glucosidase is located after alanine at position 24. The algorithm of Von Heijne predicts, for both human and rabbit isomaltase, several possible signal peptide cleavage sites with equal probability.

In chapter 4 it is shown that the signal peptide of acid α -glucosidase is not cleaved off in an *in vitro* translation system with dog pancreas microsomes in which signal peptidase is active. Conform this observation, also the acid α -glucosidase precursor synthesized in transiently transfected COS-1 cells was found to be membrane bound and to contain, by sequence analysis, an uncleaved signal peptide. Membrane association of the precursor has been demonstrated also in cultured human fibroblasts and in human placental tissue (Tsuji & Suzuki 1987; 1988). Therefore, we consider the initial retention of the signal peptide as a natural event, and we propose that the acid α -glucosidase precursor is transported from ER to Golgi complex as a type II transmembrane glycoprotein. No other lysosomal enzyme precursors are known to have uncleavable signal peptides. It is described in chapter 4 that at least part of the precursor pool is released from the membrane by proteolytic cleavage at amino acid position 29 and that this part enters the secretory pathway. The majority of the precursor molecules is transported to the lysosomes and processed to mature enzyme. Furthermore, it is shown in chapter 4 that also the sucrase-isomaltase precursor is associated with cellular membranes via its signal peptide and, in chapter 8 that this type of anchorage ensures transport to the plasma membrane.

3.2 Proteolytic processing of acid α -glucosidase and sucrase-isomaltase

Several stages can be distinguished in the maturation of acid α -glucosidase from early precursor to functional lysosomal enzyme. They are illustrated schematically in figure 1. In chapter 4 it is demonstrated that the membrane bound precursor with uncleaved signal peptide is catalytically inactive. But once the precursor is released from the membrane (and secreted into the medium) it does exhibit hydrolytic activity for α -1,4 glycosidic linkages as occurring in maltose and glycogen. However, the affinity for the macromolecular natural substrate glycogen is relatively low compared to the activity for the low molecular substrate analogue 4-methylumbelliferyl- α -D-glucopyranoside. The affinity for glycogen increases when the 110 kDa precursor is converted to the long lived 95 kDa intermediate and finally to the mature enzyme species of 76 kDa. It is suggested that the amino- and carboxyl terminal propeptides hinder the macromolecular substrate glycogen in gaining access to the substrate binding or the catalytic site of acid α -glucosidase. A similar model

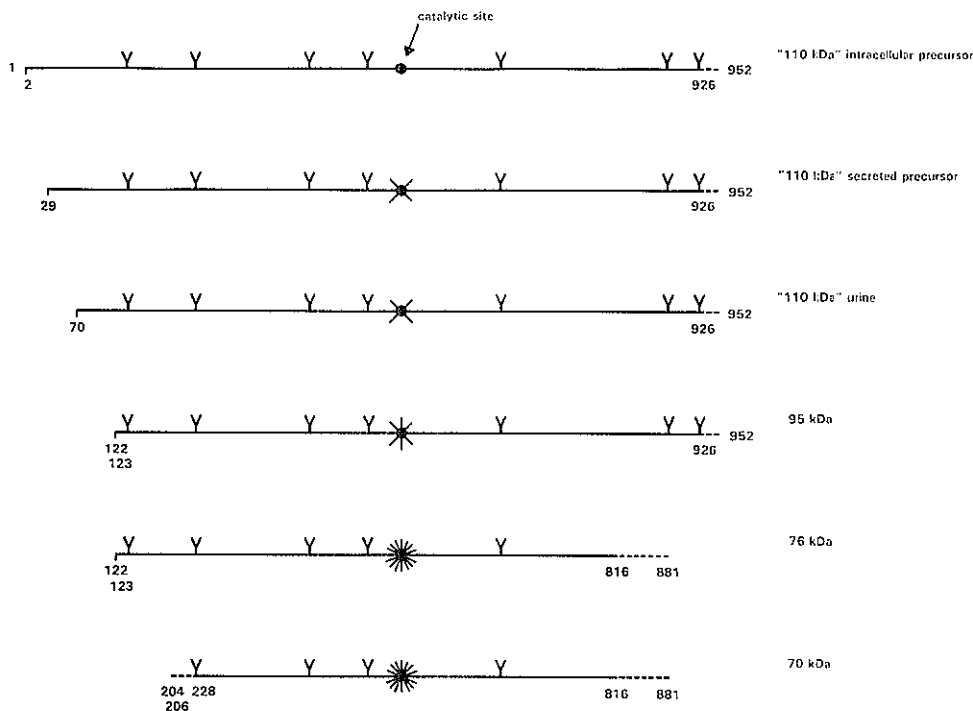


Figure 1. Schematic representation of the proteolytic processing and activation of acid α -glucosidase. The glycosylation sites are indicated (Y) and the amino acid residues at the processing sites are numbered.

was proposed by Oude Elferink et al., (1984) based on their observation that a particular monoclonal antibody raised against acid α -glucosidase did not bind to the early, unmodified precursor of 110 kDa, but did recognize the 95 kDa, 76 kDa and 70 kDa processed forms of acid α -glucosidase. Thiol proteinases are involved in the late processing of α -glucosidase since maturation is blocked by leupeptin and E64 (Oude Elferink et al., 1984; chapter 4).

From a functional point of view there is no compelling reason for synthesis of acid α -glucosidase in a catalytically inactive form. The enzyme does not hydrolyse peptide bonds but is restricted in its action to the hydrolysis of glucosidic linkages. Besides, there are already two other glycosidases operating in the endoplasmic reticulum. These enzymes, α -glucosidases I and II, are responsible for the first steps

of N-linked carbohydrate modification. They remove sequentially the outermost α -1,4 glucose and the two inner α -1,3 glucose residues from the initial $\text{Glc}_3\text{Man}_9\text{GlcNac}_2$ oligosaccharide (see chapter 1.2 and 5).

Despite the structural homology of sucrase-isomaltase with acid α -glucosidase, no proteolytic processing is involved in the biosynthesis of sucrase-isomaltase. The enzyme is synthesized as a membrane bound polypeptide of 200 kDa (chapter 4). Addition of sugar chains in the endoplasmic reticulum enlarges the molecular mass with approximately 40 kDa in COS-1 cells (chapter 8). The enzyme is transported to the plasma membrane and partially to the lysosomes (chapter 8). Upon arrival in the intestinal lumen, the precursor is cleaved into its two subunits sucrase and isomaltase which two enzymes share substrate specificity with lysosomal α -glucosidase and are similarly inhibited by acarbose and (deoxy)nojirimycine (chapter 5).

3.3 Glycosylation of acid α -glucosidase and sucrase-isomaltase

Along with proteolytic processing oligosaccharide restructuring occurs during enzyme maturation. The 110 kDa membrane bound α -glucosidase precursor is N-glycosylated at all seven potential glycosylation sites (chapter 6). Six of the seven sites can be deleted individually without effect on enzyme transport and function. But the acid α -glucosidase precursor is trapped in the endoplasmic reticulum when the second glycosylation site at Asn-233 is deleted and is subsequently degraded. This could be due to proteolytic attack at a site normally protected by the sugar chain. The same effect is observed when the glycosylation is completely prevented with the drug tunicamycin (chapter 4). Inhibition of the ER trimming glucosidases I and II by deoxynojirimycin causes a delay in α -glucosidase maturation (chapter 5). At least two of the seven N-linked carbohydrate side chains are phosphorylated enabling the enzyme to bind to the mannose 6-phosphate receptor (chapter 6). Unphosphorylated carbohydrate chains are trimmed by Golgi mannosidases and rebuilt by sugar transferases giving rise to complex type of sugar structures. The extent of sugar chain modification is determined by the length of time the protein spends in the Golgi complex. This is demonstrated in chapter 4 and 6 describing the biosynthesis of acid α -glucosidase in transfected COS-1 cells. When exit of the acid α -glucosidase precursor from the trans Golgi cisternae was prevented with the drug brefeldin A, a precursor rich in complex type of sugars was formed. Restructuring of high mannose to complex type of sugar chains also occurs in cell lines like the CaCo-2 colon adenocarcinoma cell line and the intestinal HT29 cell line with a low natural rate of

acid α -glucosidase transport (Klumperman et al., 1991; H. Bril-Luteijn, personal communication).

Human and rabbit sucrase-isomaltase have 18 and 19 N-linked glycosylation sites, respectively, but the number of sites that is actually used is as yet unknown. Our studies described in chapter 8 point out that none of the high mannose chains acquires the mannose 6-phosphate marker specific for lysosomal targeting. A fusion protein consisting of N-terminal human isomaltase and carboxyl-terminal acid α -glucosidase did not acquire the recognition marker either, but this was attributed to degradation of the fusion protein in the endoplasmic reticulum. Sugar chain modifications occur in the Golgi complex and are reflected in a gain of apparent molecular mass (Hunziker et al., 1986; Naim et al., 1988; Chantret et al., 1992; chapter 8).

3.4 Intracellular transport of acid α -glucosidase and sucrase-isomaltase

Acid α -glucosidase and sucrase-isomaltase have a relative high amino acid homology, but have distinct subcellular sites of action. The former enzyme is predominantly present in the lysosomes, whereas the latter is primarily located at the plasma membrane (see chapters 4 and 8). The targeting of both enzymes is, however, not absolutely organelle specific. Sucrase-isomaltase was demonstrated immunocytochemically to be localized partly in the lysosomes of intestinal epithelium and CaCo-2 cells (Fransen et al., 1985; Matter et al., 1990) and in the lysosomes of transiently transfected COS-1 cells (chapter 8). Acid α -glucosidase was demonstrated to line the microvilli of the intestinal brush border, of CaCo-2 cells, and of renal proximal tubule cells (Fransen et al., 1988; Willemsen et al., 1991; Klumperman et al., 1991; Oude Elferink et al., 1989). The routing of acid α -glucosidase to the plasma membrane of transiently transfected COS-1 cells is described in chapter 4. The missorting of sucrase-isomaltase to the lysosomes and α -glucosidase to the cell membrane may be inherent to the similarity of their protein structure. Sucrase-isomaltase is a typical plasma membrane protein like other intestinal brush border disaccharidases such as dipeptidylpeptidase-IV and aminopeptidase-N. If we assume that these proteins lack retention signals they would follow the default pathway and end up automatically at the plasma membrane where they are retained by their uncleaved signal peptide. Similarly, when acid α -glucosidase precursor molecules with uncleaved signal peptide escape capture by the mannose 6-phosphate receptor they too will be transported to the plasma membrane. The plasma membrane is in

continuous contact with the endosomal system enabling exchange of integral membrane proteins between both compartments. This way the lysosomal enzyme acid phosphatase and some of the lysosomal membrane glycoproteins are routed from the plasma membrane to the early endosomes and are from there shuttled to the lysosomes. Evidence has been presented that sucrase-isomaltase is transported in part via this endosomal system to the lysosomes. But, approximately 9% of newly synthesized sucrase-isomaltase gains access to the lysosomal system via a direct route. The mechanism of transport along this direct route is unknown but cannot be mediated by the mannose 6-phosphate receptor since the enzyme is not phosphorylated (chapter 8).

The transport of sucrase-isomaltase and α -glucosidase in polarized cells is even more complicated (chapter 7). The localization of sucrase-isomaltase is restricted to the apical membrane domain of intestinal epithelial cells and acid α -glucosidase co-localizes with this apical marker. The sorting signal that drives both proteins to the apical as opposed to the basolateral plasma membrane is as yet unresolved. Some alternative mechanisms of polarized transport are discussed in chapter 1.7. At present, carboxyl-terminal glycosyl-phosphatidylinositol (GPI) anchoring is the only known mechanism for apical transport of plasma membrane proteins. But this mechanism does not apply to sucrase-isomaltase nor to other enzymes with an amino-terminal signal peptide anchor. As a consequence, there is no reason to assume that a GPI anchor would be instrumental in the apical localization of acid α -glucosidase being structurally homologous to sucrase-isomaltase (chapter 7). Some suggestive evidence has been presented that the apical targeting signal of aminopeptidase-N is not localized in the amino-terminal signal peptide anchor but in the luminal domain of the protein (Vogel et al., 1992). By analogy a similar intrinsic apical sorting signal could be imprinted in the structural conformation of sucrase-isomaltase and the homologous acid α -glucosidase. The results described in chapter 7 dealing with the biosynthesis of α -glucosidase in the polarized kidney derived cell lines MDCK-II and LLC-PK₁ can be explained as to support the model of signal dependent apical targeting. A strong preference for apical transport of acid α -glucosidase (followed by secretion) was noted. However, also the lysosomal enzyme β -hexosaminidase was secreted with preference from the apical cell surface whereas this enzyme has no structural nor functional relation to intestinal brush border hydrolases. Therefore, we have proposed in figure 2 and in chapter 7 an alternative model for the apical targeting of sucrase-isomaltase, α -glucosidase and other type II glycoproteins of the apical domain of intestinal epithelial cells. In this model apical transport is supposed to occur by "selective default".

3.5 Concluding remarks

The experimental work described in this thesis contributes to the basic understanding of the biosynthesis and function of acid α -glucosidase and sucrase-isomaltase under normal circumstances. The results obtained are expected to facilitate the interpretation of abnormal enzyme transport and function in the various clinical forms of glycogenosis type II. Optimal information on the structure and function of the natural and recombinant forms of acid α -glucosidase is of further significance for the potential application of enzyme replacement therapy in glycogenosis type II.

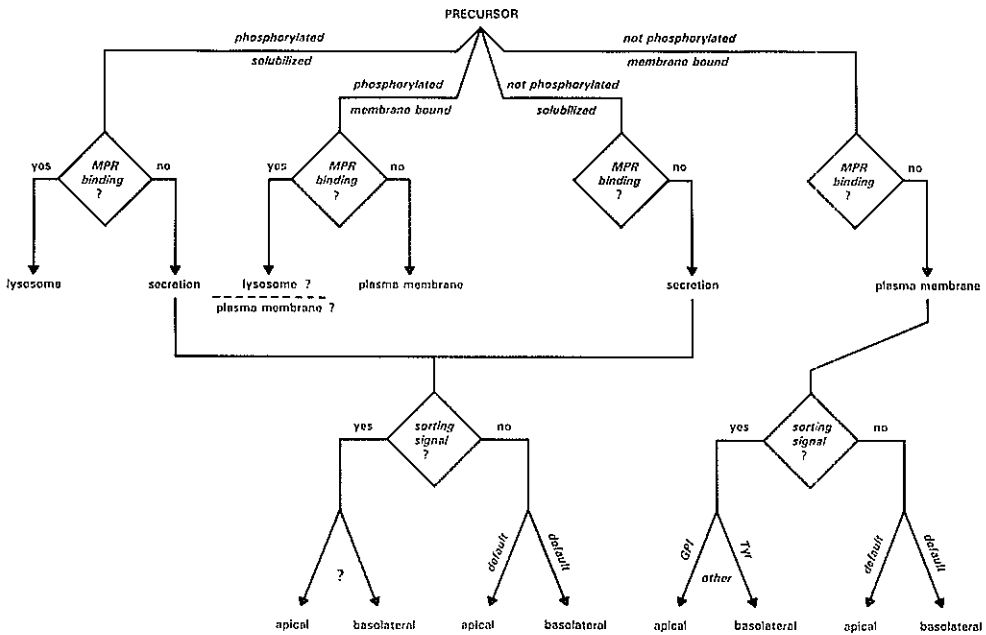


Figure 2. Flow diagram representing intracellular sorting in polarized cells. Currently known signals instrumental in sorting are indicated in *italics* and in the decision rhombi.

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Chapter 4

Structural and functional changes of lysosomal acid α -glucosidase during intracellular transport and maturation¹

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Summary

The synthesis and posttranslational modification of lysosomal acid α -glucosidase were studied in a cell free translation system and in mammalian cells transfected with acid α -glucosidase cDNA constructs. The newly synthesized precursor, sequestered in the endoplasmic reticulum, was demonstrated to be membrane bound by lack of signal peptide cleavage, and to be catalytically inactive. Sugar chain modification was shown to occur in the Golgi complex and to be dependent on the rate of transport. From the trans Golgi network different routes were found to be followed by acid α -glucosidase. A fraction of precursor molecules, proteolytically released from the membrane anchor, appeared to enter the secretory pathway and was recovered from the cell culture medium in a catalytically active form. A second fraction was transported to the lysosomes and was trimmed in a stepwise process at both the amino- and carboxyl-terminal ends. The intramolecular cleavage sites were determined. Involvement of thiol proteinases was demonstrated. Specificity for the natural substrate glycogen was gained during the maturation process. The phosphomannosyl receptor is assumed to be instrumental in the lysosomal targeting of acid α -glucosidase, but a phosphomannosyl receptor independent transport of membrane bound precursor molecules to the lysosomes, either directly or via the plasma membrane, cannot be excluded.

¹Adapted from J. Biol. Chem. 268: 2223-2231 (1993).

Introduction

Acid α -glucosidase (acid maltase; EC 3.2.1.3) degrades lysosomal glycogen to glucose (Rosenfeld, 1975). Glucose is transported out of the lysosome and reutilized in the cytoplasm (Mancini et al., 1990; Jonas et al., 1990). This catabolic pathway is particularly essential for the mobilization of glycogen in the neonatal liver (Jézéquel et al., 1965; Phillips et al., 1967). At later stages of life the main function of acid α -glucosidase seems to be the prevention of glycogen storage in the lysosomes. Inherited enzyme deficiency leads to glycogen storage disease type II (Pompe's disease), one of the over 30 different lysosomal storage diseases (Hers, 1963).

Lysosomal enzymes have common characteristic features. They have an amino-terminal signal peptide for cotranslational transport into the lumen of the endoplasmic reticulum where they are glycosylated. Carbohydrate modification occurs in this and the following Golgi compartment (Kornfeld and Kornfeld, 1985). The main transport route leads from the trans Golgi network directly to the late endosomes and lysosomes, but some lysosomal proteins are routed to the plasma membrane and reach the lysosomes via the endocytotic pathway. Three different mechanisms for lysosomal targeting are known at present. Most soluble proteins acquire the mannose 6-phosphate recognition marker and depend for their transport on the phosphomannosyl receptor (Creek and Sly, 1984; Von Figura and Hasilik, 1986; Kornfeld, 1987). Acid phosphatase lacks this recognition marker but is synthesized as a transmembrane protein and reaches the lysosomes via the plasma membrane (Waheed et al., 1988; Braun et al., 1989). Efficient endocytosis is effectuated by a tyrosine residue in the cytoplasmic carboxyl-terminal tail (Peters et al., 1990). Lysosomal membrane glycoproteins have a similar tyrosine motif, with the exception of Ig-p-85 (Fujita et al., 1991). The lysosomal glycolipid degrading enzyme glucocerebrosidase uses a third targeting mechanism which seems to involve membrane association (Van Dongen et al., 1985; Rijnbout et al., 1991).

Most lysosomal enzymes undergo limited proteolysis during and after transport. It may be restricted to cleavage of the signal peptide in the endoplasmic reticulum, but it can also be more extensive and involve additional cleavage at internal sites, or the loss of amino- and carboxyl-terminal propeptides. Some proteases are activated this way (reviewed by Neufeld (1991) and Hasilik (1992)).

Acid α -glucosidase has the typical features of a lysosomal protein, but has in addition some unusual characteristics. The enzyme is not restricted in its localization to the lysosome, it is also located at the apical surface of epithelial cells and at the plasma membrane of COS-1 cells transiently transfected with acid α -glucosidase cDNA (Fransen et al., 1988; Oude Elferink et al., 1989; Hoefsloot et al., 1990; Willemsen et al., 1991; Klumperman et al., 1991). The *ectopic* localization could possibly be related to the structural homology of acid

α -glucosidase and the brush border enzyme sucrase-isomaltase (Hoefsloot et al., 1988). A second unusual feature of acid α -glucosidase is the expression in fibroblasts from patients with I-cell disease. In contrast to the severe deficiency of other lysosomal enzymes which depend for transport on the mannose 6-phosphate receptor, the activity of acid α -glucosidase can be close to normal. Tsuji and Suzuki (1987) have suggested that acid α -glucosidase is retained in I-cell fibroblasts because of membrane association.

In the present study we have clarified the mode of membrane association of acid α -glucosidase and we have established the intramolecular and intracellular sites of proteolytic processing. Activation of the enzyme is demonstrated to occur by sequential modifications of the acid α -glucosidase precursor along the various transport routes.

Results

Proteolytic processing, glycosylation and transport of acid α -glucosidase in COS-1 cells.

COS-1 cells transiently transfected with the full length acid α -glucosidase cDNA construct (pSHAG 2) (Hoefsloot et al., 1990) were used to study enzyme biosynthesis. The same posttranslational modification events were observed as in human fibroblasts. The precursor detectable after a one hour labelling period (0 h of chase) is a polypeptide of approximately 110 kDa (fig. 1). The processing that occurs within the next 20 hours involves the formation of a 95 kDa intermediate which is subsequently converted to a 76 kDa mature species. Part of the 110 kDa precursor pool is secreted into the medium. Three days after transfection the intracellular localization of acid α -glucosidase is typically lysosomal (fig. 2A).

Synthesis of acid α -glucosidase in the presence of tunicamycin, an inhibitor of N-linked glycosylation, yields a polypeptide of approximately 97 kDa, which is degraded during the 20 h of chase (fig. 1). The unglycosylated precursor is apparently trapped in the rough endoplasmic reticulum since a perinuclear localization is observed under these circumstances (fig. 2B). This explains why the precursor is not secreted in the presence of tunicamycin (fig. 1).

The ionophore monensin and the weak base NH_4Cl do not affect the synthesis of the precursor nor the formation of the 95 kDa intermediate but they virtually block conversion of the 95 kDa intermediate to the mature 76 kDa species (fig. 1). There is no effect on secretion (fig. 1), and a normal lysosomal localization of acid α -glucosidase was obtained by immunocytochemistry (data not shown). Figure 2C illustrates the effect of the fungal metabolite brefeldin A on the transport of acid α -glucosidase. The drug causes the endoplasmic reticulum and the Golgi complex to fuse to a single compartment from which proteins cannot exit. Under these circumstances the secretion is blocked and acid α -

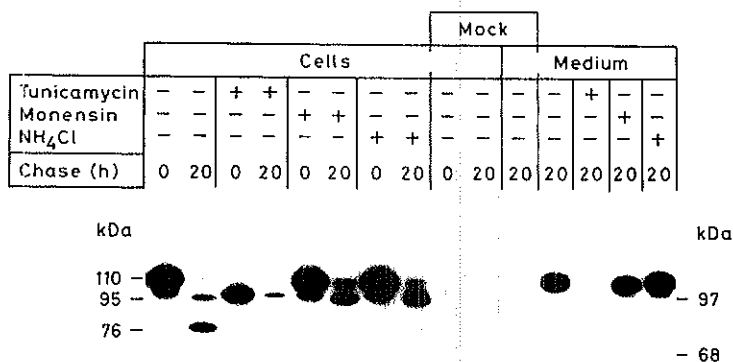


Figure 1. Biosynthesis of acid α -glucosidase in transiently transfected COS-1 cells. Acid α -glucosidase was labelled for 1 h by incorporation of ^3H leucine and immunoprecipitated either directly (0 h of chase) or after a 20 h chase from the cell homogenate. The secreted enzyme was immunoprecipitated from the medium after 20 h of chase. As a negative control, COS-1 cells were transfected with a cDNA construct of *E. coli* β -galactosidase (Mock). Tunicamycin, monensin and NH_4Cl were added as indicated.

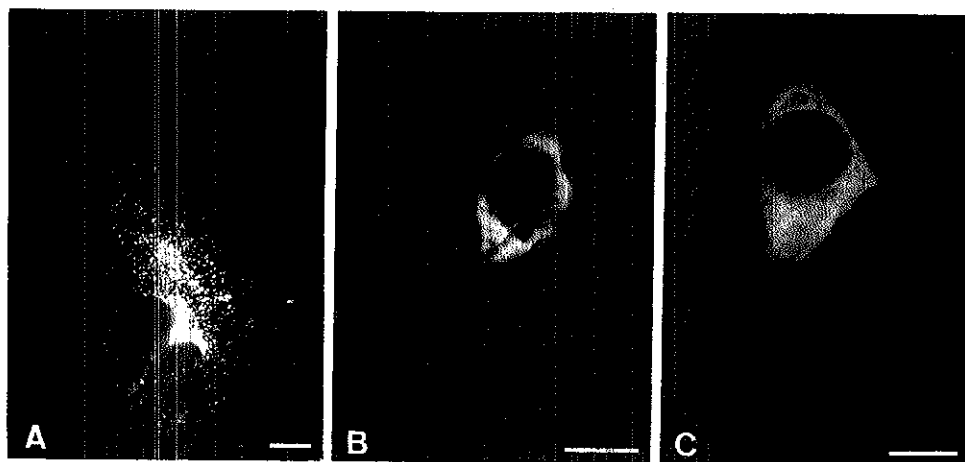


Figure 2. Immunocytochemical localization of acid α -glucosidase in transfected COS-1 cells. Cells were fixed and stained with a polyclonal antiserum directed against acid α -glucosidase as described in Materials and Methods. (A) Lysosomal localization of acid α -glucosidase at 2 days after transfection. Perinuclear localization of acid α -glucosidase in the presence of tunicamycin (B) and brefeldin A (C). Bar represents 10 μm .

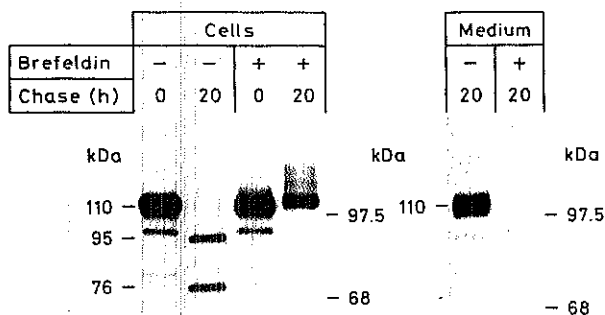


Figure 3. Biosynthesis of acid α -glucosidase in the presence of brefeldin A. The experiment was performed as described in the legend of fig. 1. Brefeldin A was added as indicated.

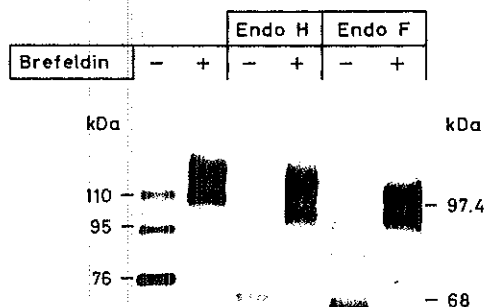


Figure 4. The glycosylation of acid α -glucosidase in the presence (+) or absence (-) of brefeldin A. Transiently transfected cells were labelled with [3 H] leucine whereafter acid α -glucosidase was analyzed as described in the legend of fig. 1. Immunoprecipitated enzyme was treated with Endo H or Endo F as indicated.

glucosidase remains in its 110 kDa precursor form indicating that the conversion from 110 kDa to 95 kDa occurs in a post-Golgi compartment (fig. 3). While trapped by brefeldin A sub-species of the precursor are formed which migrate more slowly than the usual 110 kDa precursor and which have a higher sensitivity toward Endo F than Endo H (fig. 4). These sub-species contain apparently more complex type and less high mannose type of carbohydrate chains.

The type of glycosylation can obscure existing differences in the molecular size of the intracellular and secreted 110 kDa precursor. This is shown in figure 5 which compares in addition a 110 kDa acid α -glucosidase precursor excreted in human urine. Without Endo H or Endo F treatment all three precursors seem to have the same molecular mass. However, after treatment with Endo F the intracellular precursor appears to be significantly larger than the medium precursor, which is in turn slightly larger than the precursor excreted in the urine. Comparing the effect of Endo H and Endo F it is evident that the intracellular precursor contains exclusively high mannose type of oligosaccharides whereas the precursors from the medium and the urine have acquired in part complex type of sugar chains.

Immuno-electron microscopy was performed to determine the intracellular site of proteolytic processing more exactly. For this purpose we made use of a monoclonal antibody (43G8) that does not recognize the 110 kDa precursor but only the 95 kDa intermediate and the 76 kDa mature form of acid α -glucosidase (Oude Elferink et al., 1984b). The electron micrographs of figure 6 (E and F) show that with this antibody the labelling is restricted to

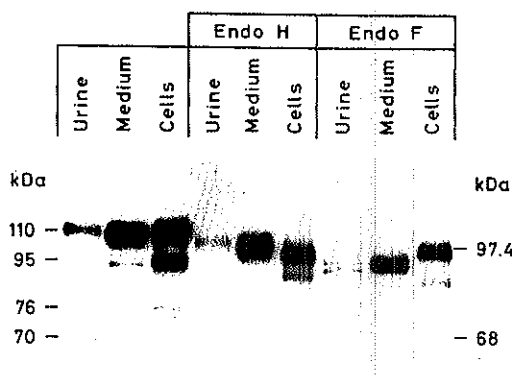
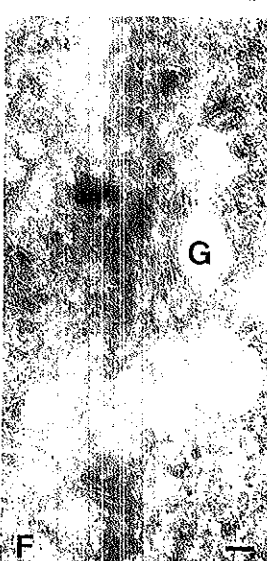
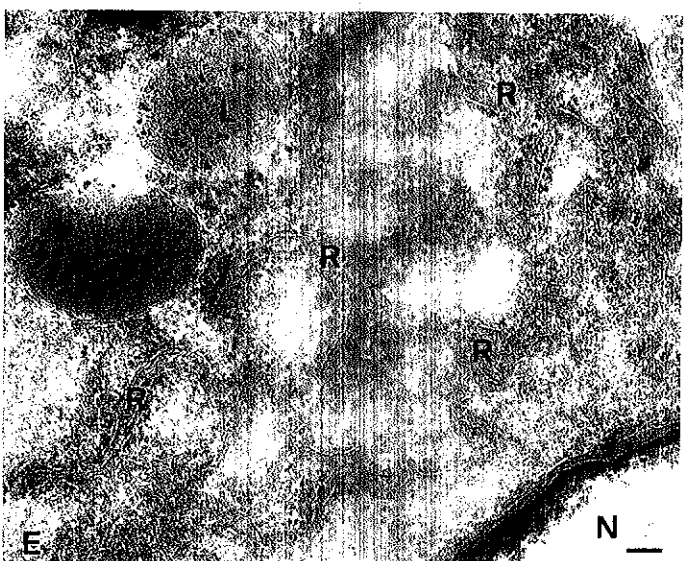
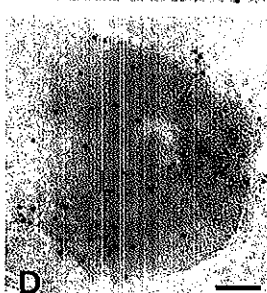
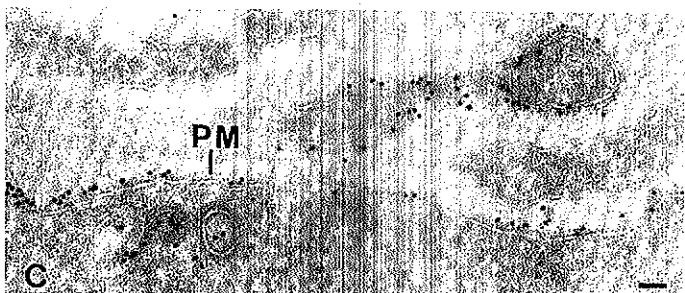
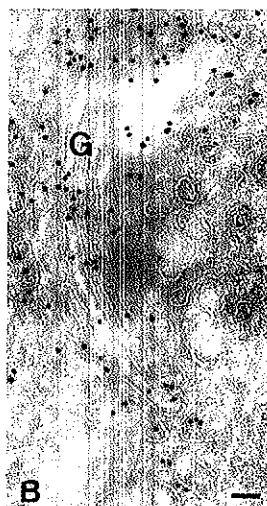
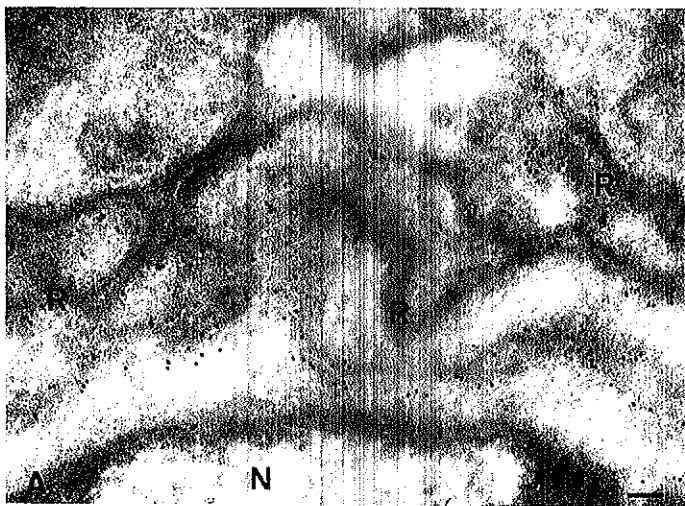


Figure 5. Comparison of the apparent molecular mass and the glycosylation of the acid α -glucosidase precursors from different sources. Acid α -glucosidase was immunopurified from transfected COS-1 cells and their media and applied to SDS-PAGE. A 110 kDa precursor of acid α -glucosidase purified from human urine was applied directly. The proteins were blotted to nitrocellulose and visualized by incubation with antibodies against acid α -glucosidase in combination with 125 I-protein A.

Figure 6. Subcellular localization of acid α -glucosidase in transfected COS-1 cells. Cryosections were prepared at 72 h after transfection and incubated with either polyclonal antisera recognizing all the different molecular forms of acid α -glucosidase (6A,B,C,D) or with monoclonal antibodies recognizing the 95, 76 and 70 kDa forms of α -glucosidase, but not the 110 kDa precursor (6E,F). N, nucleus; R, endoplasmic reticulum; G, Golgi complex; L, lysosome; P, plasma membrane. Bar represents 0.1 μ m.



lysosomes. In contrast, not only the lysosomes but also the nuclear envelope, the endoplasmic reticulum, the Golgi complex and the plasma membrane are labelled when a polyclonal antiserum is used that recognizes in addition to the processed forms the 110 kDa precursor. It thus appears that the 95 kDa intermediate is formed in the late endosomes/lysosomes.

A range of proteinase inhibitors was used to investigate the type of proteinase involved in the posttranslational modifications of acid α -glucosidase (fig. 7). The proteolytic processing of the 110 kDa precursor was delayed in cells treated with leupeptin and E64, revealing the existence of a normally short lived 100 kDa intermediate. Maturation from 95 kDa to 76 kDa was completely abolished. The 100 kDa intermediate originates from proteolytic processing rather than from oligosaccharide modification (results not shown). Incubation with 5 μ M NEM (fig. 7), which is also a thiol proteinase inhibitor like leupeptin and E64 did not interfere with the proteolytic processing of acid α -glucosidase. Higher concentrations of this inhibitor could not be tested because of their toxic effect on the COS-1 cells. Other proteinase inhibitors with different specificities like PMSF, pepstatin and 1,10 phenanthroline did not have an effect on maturation. The lysosomal localization of acid α -glucosidase was not affected by leupeptin or E64 (results not shown).

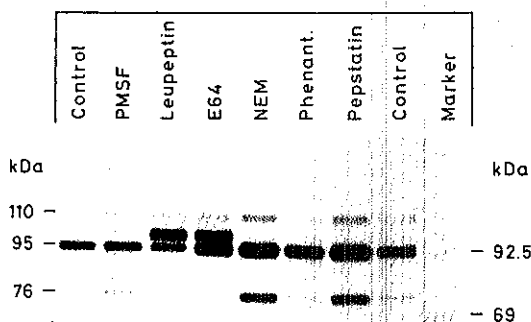


Figure 7. The effect of proteinase inhibitors on the proteolytic processing of acid α -glucosidase. Labelling and immunoprecipitation was carried out as described in the legend of fig. 1. Proteinase inhibitors were present 1 h prior to and during labelling.

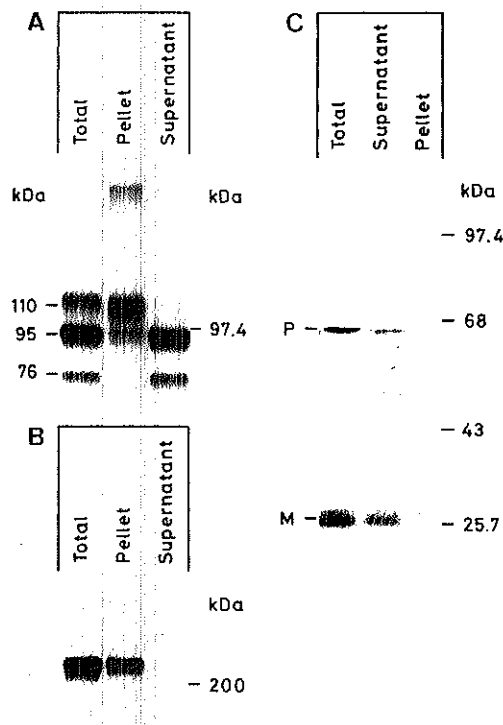


Figure 8. Membrane association of human acid α -glucosidase, rabbit sucrase-isomaltase and the human β -hexosaminidase B subunit. COS-1 cells were transfected with full length cDNA constructs coding for acid α -glucosidase (A), sucrase-isomaltase (B) and β -hexosaminidase (C) and a carbonate extract according to Klionsky and Emr (1989) was made. The respective enzymes were immunoprecipitated from the total cell homogenate (Total), the 30,000 x g membrane pellet and the remaining supernatant. The immunoprecipitated proteins were analyzed by SDS-PAGE and visualized via immunoblotting (see legend of fig. 5). P, precursor; M, mature.

Membrane-association of acid α -glucosidase.

Three methods were used to analyze the suggested (Tsuji and Suzuki, 1987) membrane association of acid α -glucosidase in different stages of maturation: (i) carbonate extraction at pH 11.0 to make membrane sheets (fig. 8), (ii) Triton X-114 phase separation (fig. 9 and 10), and (iii) saponin extraction of microsomes (fig. 11). In figure 8A the transfected COS-1 cells were extracted with carbonate, and acid α -glucosidase was immunoprecipitated from the Triton X-100 extracted membrane pellet and the 30,000 x g supernatant. Rabbit sucrase-isomaltase expressed in transiently transfected COS-1 cells was used as a typical example of a membrane bound enzyme (fig. 8B), and β -hexosaminidase as example of a soluble enzyme (fig. 8C). The 110 kDa acid α -glucosidase precursor and the

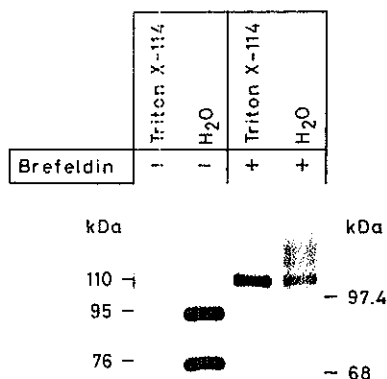


Figure 9. Triton X-114 phase separation of the different molecular forms of acid α -glucosidase expressed in transiently transfected COS-1 cells. Cells were pulse-labelled for 2 h and chased for 16 h in the presence or absence of brefeldin A, whereafter a Triton X-114 phase separation was carried out according to Bordier (1981). The detergent phase and water phase are indicated.

260 kDa rabbit sucrase-isomaltase precursor were recovered in the pellet, but the 95 kDa and 76 kDa acid α -glucosidase species in the supernatant. Both the precursor and the mature forms of β -hexosaminidase were soluble.

In an alternative procedure transfected cells were treated with brefeldin A and extracted with Triton X-114. Figure 9 shows that the accumulated precursor with complex type sugar chains (molecular mass higher than 110 kDa) segregated in the water phase, whereas the 110 kDa precursor with high mannose type of sugar chains was predominantly present in the Triton X-114 phase. Without brefeldin A the 110 kDa precursor matures to 95 kDa and 76 kDa forms which were recovered in the water phase. Additional experiments in which tunicamycin was used to trap the early acid α -glucosidase precursor in the rough endoplasmic reticulum (fig. 10A) indicated that the major pool of newly synthesized enzyme was membrane bound. Only a minor part of the acid α -glucosidase precursor pool was soluble. Rabbit sucrase-isomaltase, taken as a marker (fig. 10B), was recovered completely in the detergent phase. Similar results were obtained when tunicamycin treated cells were extracted with saponin (fig. 11). Both sucrase-isomaltase (fig. 11B) as well as the acid α -glucosidase precursor (fig. 11A) fractionated in the pellet and were not extractable with saponin.

The membrane-association of acid α -glucosidase was also studied by *in vitro* translation in the presence of dog pancreas microsomes. The translocated glycosylated precursor of approximately 110 kDa was not extractable from the 100,000 x g membrane pellet with saponin, but was extracted with Triton X-100. The unglycosylated 97 kDa *in vitro* precursor was recovered from the 100,000 x g supernatant, demonstrating that the polypeptide itself was not insoluble (fig. 12). The above results prove unequivocally that the acid α -glucosidase precursor is membrane bound.

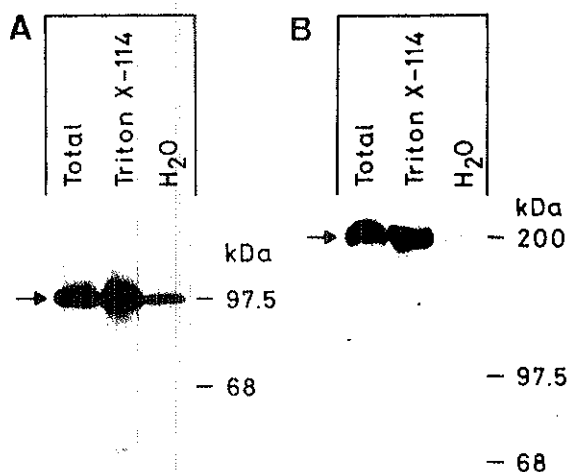


Figure 10. Membrane association of the early unglycosylated precursor of acid α -glucosidase and sucrase-isomaltase. A Triton X-114 phase separation was performed at 60 h after transfection. Tunicamycin was present from the time of transfection. Acid α -glucosidase (A) and sucrase-isomaltase (B) were immunoprecipitated from the detergent (Triton X-114) and water phase as well as from the total cell homogenate. The enzymes were visualized on immunoblots after SDS-PAGE as described in fig. 8.

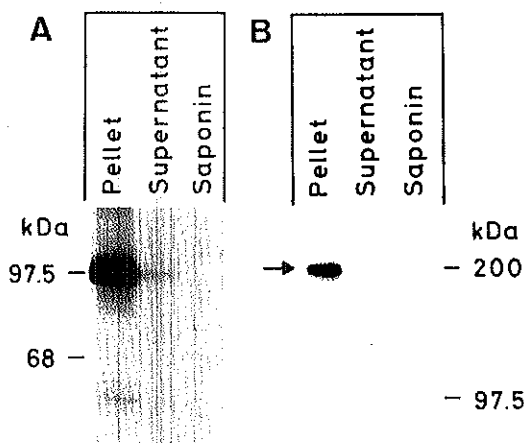


Figure 11. Membrane association of the early unglycosylated precursor of acid α -glucosidase and sucrase-isomaltase demonstrated with saponin. After transfection with full length acid α -glucosidase cDNA (A) or sucrase-isomaltase cDNA (B) cells were disrupted and a 100,000 x g pellet was prepared. The supernatant was kept. The pellet was extracted with 0.2% saponin. Acid α -glucosidase or sucrase-isomaltase were immunoprecipitated from the supernatant, the saponin washes and the Triton X-100 extract of the pellet after saponin wash. Analysis was performed by immunoblotting.

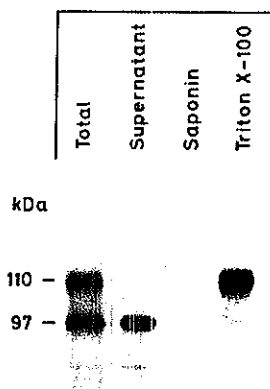


Figure 12. Membrane association of the glycosylated precursor of acid α -glucosidase demonstrated by *in vitro* translation. Acid α -glucosidase mRNA was translated in a reticulocyte lysate in the presence of dog pancreas microsomes. Microsomes were spun down at 100,000 x g, they were washed three times with saponin and subsequently extracted with Triton X-100. Acid α -glucosidase was immunoprecipitated from the different fractions and analysed as in fig. 11.

The possibility was investigated that the amino-terminal signal peptide of acid α -glucosidase could act as membrane anchor. To this end truncated cDNA constructs coding for the amino terminal ends of acid α -glucosidase and rabbit sucrase-isomaltase (RIS) were used for *in vitro* transcription and translation. The constructs were made in such a way that the truncated polypeptide contained one glycosylation site to mark translocation across the microsomal membrane. The glycosylated peptide consisting of the first 174 amino-terminal residues of acid α -glucosidase was recovered in the saponin extracted 100,000 x g microsomal pellet and was solubilized with Triton X-100 (fig. 13). In addition, the glycosylated 160-residue amino-terminal peptide of sucrase-isomaltase fractionated in the pellet. The translocated but deglycosylated peptide (Endo F treatment) and the unglycosylated peptide of acid α -glucosidase (synthesized in the absence of microsomes) were of the same size. This indicates that the signal peptide is not cleaved off. The same holds for sucrase-isomaltase, but β -lactamase (used to test for signal peptidase activity) lost its signal peptide and was solubilized as a result.

Amino acid sequence analysis.

The different molecular forms of acid α -glucosidase were isolated in order to determine the exact sites of proteolytic processing. The secreted precursor was immunoprecipitated from the medium of transiently transfected COS-1 cells and stably transfected BHK cells. The intracellular membrane bound precursor was immunopurified from the Triton X-100 extract of a saponin washed 100,000 x g pellet of transfected COS-1 cells. The 95 kDa intermediate and the 76 kDa and 70 kDa mature proteins were recovered from cell homogenates by immunoprecipitation and separated by SDS-PAGE. Amino terminal

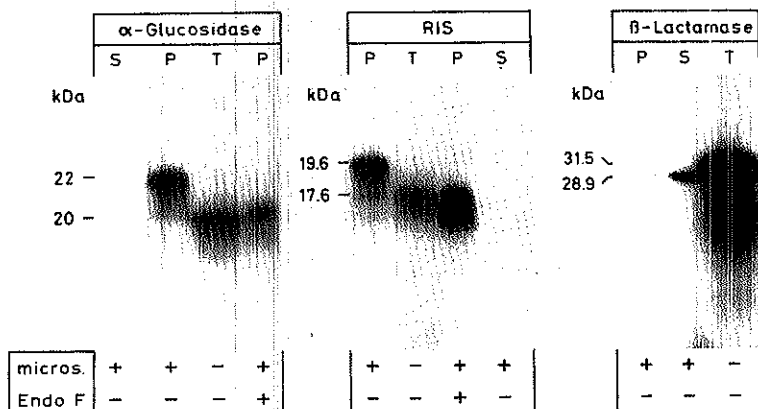


Figure 13. Membrane association of amino-terminal peptides of acid α -glucosidase and rabbit sucrase-isomaltase (RIS) studied by *in vitro* translation of truncated constructs. Truncated cDNA's were transcribed and translated *in vitro* in the presence of microsomes when indicated. The membrane association was analyzed as described in the legend of fig. 12. Endo F treatment was performed after immunoprecipitation. T: immunoprecipitation from the total translation reaction; S: saponin wash of 100,000 x g pellet; P: Triton X-100 extract of pellet after saponin wash.

sequence analysis was performed after blotting, using an Applied Biosystems 473 A protein sequenator. The sequences were compared with those of the acid α -glucosidase precursor previously isolated from human urine and the 95, 76, and 70 kDa forms of α -glucosidase purified from human placenta (Hoefsloot et al., (1988) and unpublished results). The sequence data are given (Table I). The proteolytic events are summarized in figure 14. The intracellular membrane bound precursor lacks the initiator methionine and starts with glycine at position 2. The signal peptide is uncleaved. The precursor precipitated from the medium initiates at residue 29 with histidine, and the precursor excreted in the urine starts at position 70 with alanine. The 95 kDa intermediate and the 76 kDa mature enzyme species expressed in BHK or COS-1 cells or isolated from placenta have the same amino terminal ends located either at position 122 (methionine) or 123 (glycine). The 70 kDa form of acid α -glucosidase from human placenta also shows molecular heterogeneity. A fraction of molecules starts at position 204 (alanine), a second fraction at position 206 (serine), whereas a third sequence begins at residue 228 (glycine). The positions of the carboxyl-terminal cleavage sites are discussed below.

Table I

N-terminal amino acid sequences of the different forms of acid α -glucosidase

Species	Sequence	Source
110 kDa membrane-bound	² G.RHPP.S.RLLA	COS-1
110 kDa medium	²⁹ HILLHDFFLLVPRELSGS.PVLEE	BHKS11
110 kDa urine	⁷⁰ AHPGRPRAVPTQXDVPNSR	human urine
95 kDa	¹¹⁹ GAQ.GQP	BHKS11 placenta placenta COS-1
	¹²² MGQPWXFFPPSYPSYKLEN	
	¹²³ GQPW	
	¹²³ GQPWXFFPP.YP..K	
76 kDa	¹²² MGQPWXFFPPSY	BHKS11 placenta BHKS11 placenta
	¹²² MGQPWXFFP	
	¹²³ GQPWXFFPP.Y	
	¹²³ GQPWX..PPSYPSY	
70 kDa	²⁰⁴ AP.PL	BHKS11 placenta placenta placenta
	²⁰⁴ APSPLYSVEFSEEP.GVIV..QLDG.V	
	²⁰⁶ SPLYSVEF.E.PFGVIVH..LD..V.L	
	²²⁸ G.VLL.T.VAPLFFADQFLQL	

X = Cysteine; amino acid residues that could not be determined with certainty are indicated with a period. The numbers in superscript refer to the position of the first amino acid residue of the listed sequence.

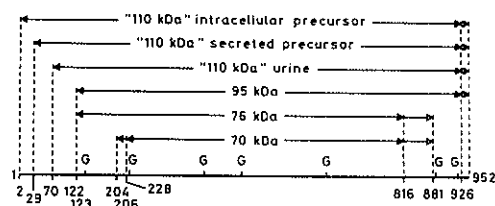


Figure 14. The proteolytic processing sites of acid α -glucosidase. The glycosylation sites are indicated (G).

Functional characteristics of acid α -glucosidase.

Conduritol β -epoxide (CBE), a substrate analogue of acid α -glucosidase, was used to distinguish between catalytically active and inactive forms of the enzyme. We have shown previously that acid α -glucosidase needs to be catalytically active to bind CBE covalently (Hermans et al., 1991). The binding causes a mobility shift in SDS-PAGE. CBE changed the mobility of the 95 kDa intermediate, the mature 76 kDa and 70 kDa species of acid α -glucosidase expressed in stably transfected BHK cells. The 110 kDa precursor secreted by these cells also appeared to be catalytically active (results not shown). The same experiment was conducted with transiently transfected COS-1 cells in which the 110 kDa precursor was more abundantly present. This intracellular membrane bound precursor did not bind CBE.

Table II

Relation between maturation and glycogen degrading activity of acid α -glucosidase. For each of the molecular forms of acid α -glucosidase the activity for both the natural (glycogen) and the artificial substrate (4-methyl-umbelliferyl- α -D-glucopyranoside; 4-MU) was measured and the ratio of the activity for glycogen over 4-MU was calculated.

Molecular Form	Ratio
110 kDa	1.5
95 kDa	5.2
76/70 kDa	10.7

To analyze the activation process, we compared for each of the different forms of acid α -glucosidase the activity for the natural substrate (glycogen) and the artificial substrate (4-methylumbelliferyl- α -D-glucopyranoside; 4-MU). The results are given in Table II. The glycogen over 4-MU ratio of the secreted precursor was measured using the medium of the stably transfected BHK cells. The 95 kDa intermediate was generated by feeding the medium precursor to fibroblasts from a patient with complete deficiency of acid α -glucosidase. The 110 kDa precursor was taken up via the mannose 6-phosphate receptor and converted to 95 kDa. Further processing to 76 kDa was inhibited by the addition of leupeptin. Mature 76 kDa and 70 kDa acid α -glucosidase forms were generated by culturing stably transfected BHK cells for 48 hours in the presence of cycloheximide. Under these conditions *de novo* synthesis

is blocked and all precursor forms are converted to mature enzyme. The data in Table II show that the ratio of glycogen over 4-MU increases as the result of proteolytic processing.

Discussion

Lysosomal enzymes are subjected to posttranslational modifications. Glycosylation renders stability to the proteins and phosphorylation of mannose residues provides a lysosomal targeting signal for soluble lysosomal proteins. Proteolytic release from the membrane spanning signal peptide sets the enzymes free for further transport to the lysosomes. Additional trimming at amino- and carboxyl-terminal ends and internal cleavage of lysosomal protein precursors have been reported. The latter can be essential for activation. We have studied the posttranslational modifications of acid α -glucosidase in greater detail to better understand the function of the enzyme and its dysfunction in glycogenosis type II, and to contribute to the general concept of glycoprotein handling and targeting.

The signal peptide as membrane anchor

Resident proteins of the lysosomes have a signal peptide like the secretory and plasma membrane glycoproteins (Blobel and Dobberstein, 1975; Blobel, 1980). The signal peptide is inserted in the endoplasmic reticulum membrane with the amino-terminal n-region extending in the cytoplasm (Shaw et al., 1988).

The amino acid sequence of acid α -glucosidase fulfills the requirements of a signal peptide. Positively charged residues are found at amino acid positions 4,5,10 and 11. Histidine and arginine present in this order at the latter two positions are likely to lie immediately adjacent to the cytoplasmic side of the membrane. The following non-polar stretch of 17 residues consists for 65% of leucine and alanine, and is predicted by the Garnier-Osguthorpe and Robson analysis (Garnier et al., 1978) to form an α -helix. This stretch ends with histidine at position 29. According to the algorithm of Von Heijne (Von Heijne (1986, 1988) there is a potential signal peptidase cleavage site after alanine at position 24 ($P[i]/P_{max} = 1$) and a second site with much lower probability between glycine at position 28 and histidine at position 29 ($P[i]/P_{max} = 0.00354$). It was this latter histidine that was found to be the amino-terminal end of the secreted α -glucosidase precursor (Table I). These data suggest that signal peptidase cleaves at the second less favorable site or that cleavage occurs by an as yet unidentified peptidase located in the endoplasmic reticulum or in a more distal compartment. For instance, a Golgi based proteinase has been implicated in the release of yeast acid phosphatase from its membrane anchor (Schönholzer et al., 1985). As an alternative the possibility has to be considered that signal peptidase does cleave at the first most favorable site (Ala 24) whereafter 4 more residues are lost by the

action of exo- or endopeptidases. Such a multistep mechanism of cleavage is, however, expected to result in microheterogeneity of the amino-terminal sequence of the secreted precursor, and this was not observed. No contaminating polypeptides starting one or more positions ahead of His 29 were discovered. Moreover, amino- and carboxyl-terminal trimming is usually a late processing event (Stirling et al., 1988; Quon et al., 1989).

Apart from the soluble precursor secreted in the medium we were able to isolate and partially sequence a membrane-bound precursor of acid α -glucosidase. Tsuji and Suzuki have reported previously on the existence of such a precursor. Membrane association via the mannose 6-phosphate receptor was excluded and suggestive evidence for membrane integration was presented (Tsuji and Suzuki 1987, 1988; Tsuji et al., 1988). With the use of three different methods discriminating between integral membrane proteins and loosely associated or soluble proteins we could demonstrate the existence of a membrane-bound acid α -glucosidase precursor unequivocally. This precursor was isolated and shown to contain an uncleaved signal peptide. The latter result is consistent with the demonstrated lack of signal peptide cleavage in the *in vitro* translation system, and the apparent skipping of the predicted cleavage site. The signal peptide of acid α -glucosidase can, thereby, function as a membrane anchor as it does for the structurally and functionally closely related enzyme sucrase-isomaltase, and for several other plasma membrane proteins and the lysosomal membrane protein lgp-85. We speculate that the reported *ectopic* localization of acid α -glucosidase in the brush border of intestinal epithelial cells, kidney proximal tubule cells, and on the plasma membrane of transfected COS-1 cells is caused by inefficient release of the precursor from the membrane anchor.

Transport of sucrase-isomaltase from the brush border membrane to the lysosomes via endocytosis has been demonstrated. Another fraction of the sucrase-isomaltase precursor molecules was shown to reach the lysosomes via a direct pathway (Matter et al., 1990). By analogy it is feasible that a membrane bound fraction of acid α -glucosidase precursor molecules reaches the lysosomes independent of the mannose 6-phosphate receptor. This would explain the near normal activity of acid α -glucosidase in I-cell disease (Van Dongen et al., 1985; Tsuji and Suzuki, 1988). The mannose 6-phosphate receptor dependent pathway will operate in parallel and ensure lysosomal targeting of acid α -glucosidase precursor molecules that are released from the membrane before exit from the trans Golgi network.

Posttranslational modifications and transport

The acid α -glucosidase precursor is N-glycosylated in the endoplasmic reticulum. We have recently demonstrated that all seven potential glycosylation sites are used but that sixth of the seven sites can be deleted individually without apparent effect. Deletion of the site at amino acid position 233 interferes with enzyme maturation (Hermans et al., 1993). Our experiments indicate that glycosylation of the precursor is essential for stabilization and

transport. When glycosylation was prevented by tunicamycin, the precursor did not exit from the endoplasmic reticulum and was degraded.

The intracellular acid α -glucosidase precursor which is formed in a one hour labelling period is equally sensitive to Endo H as to Endo F and has, therefore, exclusively high mannose type of carbohydrate chains. At least two of the seven chains are phosphorylated (Hermans et al., 1991). Phosphorylation is assumed to block further modification, but some of the remaining carbohydrate chains are apparently rebuilt to complex type of structures. The processed secreted precursor has a different Endo H sensitivity compared to the membrane-bound intracellular precursor. The intracellular processed forms, of lower molecular mass, also contain complex type of sugar chains. It is of interest to note that the degree of complex glycosylation is not imprinted in the protein, but dependent on the length of sojourn in the Golgi cisternae. When exit from the Golgi complex is prevented by brefeldin A, the degree of complex glycosylation increases. This is reflected in an increase of the apparent molecular mass. The rate of acid α -glucosidase transport through the Golgi complex must be relatively fast, since only limited complex glycosylation is observed in the absence of brefeldin A.

Until the moment that the secretory and lysosomal pathways diverge in the trans Golgi network, no proteolytic processing of the newly synthesized acid α -glucosidase seems to have occurred other than the cleavage of the first 28 amino terminal residues that are no longer present in the secreted precursor. The following processing steps must occur in more distal compartments. The sequence data obtained indicates that the long lived 95 kDa intermediate is 92-93 residues shorter at the amino terminal end than the secreted precursor with cleaved membrane anchor (fig. 14). We do not know whether this intermediate has undergone additional carboxyl-terminal trimming. But if so, it does not involve more than 26 residues, since the seventh glycosylation site at Asn 925 is not lost in the process (Hermans et al., 1993). The formation of the 95 kDa intermediate is not dramatically affected by NH_4Cl or monensin, but leupeptin and E64 have an inhibitory effect. In the presence of these thiol proteinase inhibitors, the existence of a short lived intermediate of approximately 100 kDa becomes apparent. This intermediate must arise by cleavage of the precursor at a site located in between the amino terminal ends of the 110 kDa precursor and the 95 kDa intermediate. The exact site remains to be determined. It is possible that this site coincides with the amino terminal end of the precursor excreted in human urine (Fig. 14) (Oude Elferink et al., 1984a; Hoefsloot et al., 1988). The involvement of thiol proteinases in the late processing of lysosomal proteins has recently been reviewed by Hasilik (1992).

Thiol proteases also appeared to be essential for the further maturation of acid α -glucosidase from 95 to 76 kDa. The sequence data show this to be a carboxyl terminal event. The amino terminal sequences of the 95 and 76 kDa species are identical. Degeneration of the sequence indicates that the polypeptides are subjected to attack by exopeptidases once

they are formed. This phenomenon has been reported for several other lysosomal enzymes (Hasilik, 1992). Unfortunately we did not succeed to determine the carboxyl terminal end of the 76 kDa enzyme exactly, but the site of proteolytic cleavage must be located in a 65-residue amino acid region extending from position 816 to 881. The amino terminal end of this region is determined by the last amino acid of a peptide that was isolated after tryptic digestion of the 76 kDa form of acid α -glucosidase from human placenta (Hoefsloot et al., 1988). The carboxyl terminal end is determined by the location of the sixth glycosylation site which is lost upon conversion of the 95 to 76 kDa species (Hermans et al., 1992).

A further proteolytic modification of acid α -glucosidase involves the conversion of the 76 kDa species to 70 kDa. Knowing the primary sequence of acid α -glucosidase and the amino terminal ends of both the 76 kDa and 70 kDa forms, we conclude that this last processing step is almost exclusively amino terminal. The 70 kDa species is apparently subjected to further proteolysis, since a second and a third amino terminal sequence of the 70 kDa preparation start, respectively, 2 and 25 amino acid residues downstream of the major sequence.

As mentioned above, all the latter proteolytic modifications must occur distal from the trans Golgi network i.e. in late endosomal or lysosomal compartments. Supportive evidence for this was obtained by the immunocytochemical localization of acid α -glucosidase with monoclonal antibodies. The monoclonal antibodies used do not bind to the 110 kDa intracellular and secreted precursor, but they do recognize the more mature species (Oude Elferink et al., 1984b). With these antibodies we obtained only lysosomal labelling, indicating that the 95 kDa and 76 kDa species are not formed before entry of the precursor in the lysosomes. The latter results strengthen and extend the conclusions of Oude Elferink et al. (1984b) about the subcellular site of acid α -glucosidase maturation in fibroblasts. The delay in maturation seen in the presence of NH_4Cl or monensin could be due to an increase of the lysosomal pH whereby the involved thiol proteinases are inactivated.

The activation of acid α -glucosidase

Some lysosomal enzymes are known to be catalytically active almost immediately after synthesis. This has for instance been demonstrated for hexosaminidase using an in vitro translation system (Sonderfeld-Fresko and Proia, 1988). Other lysosomal proteins are activated by proteolytic cleavage. Among this group are the cathepsins (see for review Hasilik, 1992). The physiological function of enzyme activation at a late stage of biosynthesis could be to prevent the deleterious action of the enzyme in a pre-lysosomal compartment. But for acid α -glucosidase having glycogen and maltose as natural substrates it is difficult to envisage what harm the enzyme could do when activated prematurely. Nevertheless, the results indicate that the early membrane bound precursor is catalytically inactive and that successive events of proteolytic maturation are required to obtain an enzyme configuration

which allows optimal activity toward the natural substrate glycogen. The uncleaved amino terminal and carboxyl terminal extensions of the precursor seem to hinder the macromolecular substrate glycogen sterically in gaining access to the substrate binding site or the catalytic site of acid α -glucosidase located within the boundaries of the mature enzyme. A similar structural conformation of the acid α -glucosidase precursor was proposed to explain the differential reactivity of monoclonal antibody 43G8 with the precursor *versus* the processed forms of acid α -glucosidase. The epitope of 43G8 was postulated to be located near the active site and to be masked by the precursor polypeptide extension (Oude Elferink et al., 1984b).

This structural model is beforehand a simplification of the real situation. The point mutation associated with the GAA2 allele of acid α -glucosidase was identified by Martiniuk et al. (1990). It concerns a Asp to Asn substitution at amino acid position 91 which is located in the amino-terminal precursor extension, 29 residues ahead of the amino-terminal end of the 76 kDa mature enzyme. Thus, the mutation is lost upon maturation. Nevertheless the mutation results in a highly increased k_m of the mature enzyme for glycogen. This phenomenon cannot be explained by the simple model of steric hindrance depicted above, but suggests the propeptide to have an early decisive effect on the final conformation of acid α -glucosidase.

The acquired information on the posttranslational modification events is essential to understand the structure-function relation of acid α -glucosidase, and will help to interpret the effect of point mutations in the acid α -glucosidase gene which lead to glycogen storage disease type II.

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We thank Dr. Ben Oostra and R. Willemsen for stimulating discussions. Brefeldin A was a generous gift of Dr. A. Takatsuki. Rabbit sucrase isomaltase cDNA was kindly provided by Dr. Giorgio Semenza and Dr. Ned Mantei, and antibodies against sucrase-isomaltase by Dr. Hans Wacker from the Laboratory of Biochemistry, ETH Zürich. Dr. John Stirling provided β -hexosaminidase cDNA. Figures and photographic artwork were prepared by Tom de Vries Lentsch, Ruud Koppenol, and Pim Visser. Jeannette Lokker provided secretarial assistance.

Materials and methods

Transfection procedures.

Monkey kidney COS-1 cells were cultured in Dulbecco's modified Eagle's medium, supplemented with antibiotics and 10% fetal calf serum, under air/CO₂ (9:1). COS-1 cells were transfected using the DEAE-dextran method exactly as described by Hoefsloot et al. (1990). The human acid α -glucosidase cDNA (pSHAG 2) (Hoefsloot et al., 1988; 1990), the rabbit sucrase-isomaltase cDNA (Hunziker et al., 1986) and the full length cDNA coding for the β -subunit of human hexosaminidase (O'Dowd et al., 1985; Stirling et al., 1988) were cloned in the eukaryotic expression vector pSG5 (Green et al., 1988). The plasmid pGA293 was used for the expression of E. coli β -galactosidase (An et al., 1982). Stably transfected baby-hamster kidney cells (BHK) were

obtained by co-transfection with pSHAG 2 and PITA (a plasmid conferring resistance to the antibiotic G418) in a molecular ratio of 9:1. For these transfections a modification of the calcium-phosphate method was used (Graham and Van der Eb, 1973). In short 20 µg DNA was added to 10^6 cells in 10 cm tissue-culture dishes as a calcium phosphate-precipitate. After 16 hours the cells were treated with 30% (v/v) Me₂SO for 30 minutes. Drug resistant cells were selected with G418 at a final concentration of 800 µg/ml. Colonies expressing acid α-glucosidase were subcloned by limiting dilution.

Biochemical assays.

Cell lysates were prepared by sonication of cell pellets in distilled water. Protein concentrations of cell homogenates were measured with the use of a bicinchoninic acid (BCA) protein assay kit (Pierce), using bovine serum albumin as standard.

Acid α-glucosidase activity was determined with the artificial substrate 4-methylumbelliferyl-α-D-glucopyranoside (Melford) or with the natural substrate glycogen as described by Koster et al., (1972) and Reuser et al., (1978). To assay the activity of the secreted 110 kDa precursor for glycogen the cells were grown in glucose free DMEM to which 2.5 mM inosine and 10% fetal calf serum (dialyzed against 10 mM sodium phosphate and 150 mM NaCl, pH 7.2) was added.

Metabolic labelling, immunoprecipitation and electrophoresis.

Transiently transfected COS-1 cells were pulse labelled for one hour with [³H] leucine (190 Ci/mmol; Amersham) three days after transfection as described by Hasilik and Neufeld, (1980) and Reuser et al., (1985). The cells were either harvested directly or after a subsequent period of 20 hours in fresh medium without radioactive precursor. Acid α-glucosidase was immunoprecipitated with rabbit polyclonal antiserum in combination with protein A Sepharose beads. Some samples were deglycosylated with Endo H or Endo F according to the instructions of the manufacturer (Boehringer). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed on 8% slab gels according to Laemmli, (1970). The radioactive polypeptides were visualized by fluorography.

Transport and proteinase inhibitors.

Inhibitors were added to the culture medium one hour prior to pulse-chase labelling. The following inhibitors were used: tunicamycin (3 µg/ml) (Boehringer); NH₄Cl (10 mM) (Merck); monensin (1 µM) (Sigma); brefeldin A (10 µg/ml) (kindly provided by Dr. A. Takatsuki); PMSF (2 mM) (Sigma); pepstatin (5 µM) (Boehringer); 1,10 phenanthroline (5 µM) (Sigma); Leupeptin (0.1 mM) (Sigma); E64 (0.1 mM) (Sigma); NEM (5 µM) (Boehringer); cycloheximide (20 µg/ml) (Sigma).

Immunoblotting.

Acid α-glucosidase was immunoprecipitated from cell homogenates and culture media with polyclonal rabbit antiserum, rabbit sucrase-isomaltase was precipitated with polyclonal antiserum raised in guinea pigs (gp 71; kindly provided by H. Wacker), and hexosaminidase with polyclonal rabbit antiserum (Reuser et al., 1985). Protein A Sepharose beads were added in each case. Some samples were at this stage deglycosylated with Endo H or Endo F. Other samples were incubated with conduritol B epoxide (CBE) for 7 hours at 37 °C according to Hermans et al., (1991) to label the active site of acid α-glucosidase. Precipitated proteins were separated by SDS-PAGE under reducing conditions (Laemmli, 1970) and subsequently blotted onto nitrocellulose filters (Towbin et al., 1979). They were visualized with the same antisera as above in combination with [¹²⁵I] labelled protein A. Blots were exposed to Kodak XAR film.

Assessment of membrane association.

Three different methods were used to determine the membrane association of acid α-glucosidase, rabbit sucrase-isomaltase and β-hexosaminidase. In the first procedure a cell homogenate was made by sonication in 50 mM Tris-HCl, pH 7.4 containing 0.3 M NaCl, 1 mM EDTA, 1 mM PMSF, and 5 mM iodoacetamide. Membranes were pelleted by centrifugation at 100,000 x g. The pellet was rinsed three times in the same buffer containing 0.2% saponin. The pellet was finally extracted with 0.5% Triton X-100. Proteins were immunoprecipitated from the first 100,000 x g supernatant, from the pooled saponin washes and from the Triton X-100 extract. As a second procedure to assess membrane association a Triton X-114 phase separation was performed according to Bordier, (1981). The third procedure involved disruption of the cells with 10 mM Na₂CO₃, pH 10.7, resulting in the release of soluble proteins, whereas integral membrane proteins remain associated with the 30,000 x g pellet (Klionsky and Emr, 1989). The pellet was finally extracted with 0.5% Triton X-100.

In vitro transcription / translation

The full length acid α -glucosidase cDNA and the rabbit sucrase-isomaltase cDNA, cloned in pSG5, were digested with Xho II and Hind III respectively. This resulted in small 5'-cDNA constructs linked to the T7 promotor in the plasmid. When these cDNA constructs are transcribed and translated they code for truncated proteins of approximately 20 kDa and contain one potential N-linked glycosylation site. mRNA transcription and translation were performed as described by Hoefsloot et al., (1990).

Amino acid sequence analysis.

The different molecular forms of acid α -glucosidase expressed in transiently transfected COS-1 cells or in stably transfected baby hamster kidney cells (BHK) were separated using SDS-PAGE (8% gel) and blotted to Problot (Applied Biosystems) as described before (Hoefsloot et al., 1988). The precursor of acid α -glucosidase excreted in the urine was purified according to Oude Elferink et al., (1984a).

To obtain carboxyl terminal peptides of the mature 76 kDa and 70 kDa forms of acid α -glucosidase the enzyme was purified from human placenta and digested with trypsin. Carboxyl terminal peptides were isolated with an anhydrotypsin column following the instructions of the manufacturer (Pierce). The peptides were dissolved in 50% acetonitrile in H₂O containing 0.1% trifluoroacetic acid. The peptides were separated by reverse-phase HPLC using a RP-8 column (Merck) and a gradient of 0 - 55 % acetonitrile.

Amino acid sequence analysis of the blotted polypeptides and trypsin fragments was performed on an Applied Biosystems model 473A protein sequenator. Sequence analysis data are given in Table I.

Immunocytochemistry.

For application of lightmicroscopy, the cells were seeded in low density on coverslips the evening before analysis and prepared for immunocytochemistry as described (Van Dongen et al., 1984). Incubations were performed with a polyclonal rabbit antiserum against human acid α -glucosidase (Reuser et al., 1985). Immune complexes were visualized with goat anti-rabbit IgG conjugated to fluorescein.

For immuno-electronmicroscopy cells were fixed in 1% acrolein and 0.4% glutaraldehyde in 0.15 M sodium bicarbonate, pH 7.4, for 1 hour at 4°C. The cells were washed with phosphate buffered saline (0.1 M sodium phosphate, pH 7.2 and 0.5 M NaCl) and gently scrapped off the culture dish with a rubber policeman. Cells were embedded in 10% gelatin. Ultrathin cryosections were immunostained by incubation with the same antibodies, followed by an incubation with goat anti-(rabbit IgG) coupled to 10 nm colloidal gold (GAR-10, Aurion). Other sections were incubated with a mouse monoclonal antibody against acid α -glucosidase (43G8; Oude Elferink et al., 1984b). Immune complexes were visualized with goat anti-(mouse IgG) coupled to 10 nm colloidal gold (GAM-10, Aurion). Control sections were incubated with pre-immune serum instead of specific antiserum.

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Chapter 5

Effects of N-hydroxyethyl-1-deoxynojirimycin (BAY m 1099) on the activity of neutral- and acid α -glucosidases in human fibroblasts and HepG2 cells¹

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Summary

The effect of the glucose analogue N-hydroxyethyl-1-deoxynojirimycin (BAY m 1099) on the activity of α -glucosidases was studied in human fibroblasts and HepG2 cells. BAY m 1099 inhibits neutral and acid α -glucosidase activities of both cell types in a dosage-dependent and reversible manner. Inhibition of endoplasmic reticulum glucosidases I and/or II is suggested by delayed processing of lysosomal (acid) α -glucosidase. Competitive inhibition of mature acid α -glucosidase leads to lysosomal accumulation of glycogen as in glycogenosis type II. There seems to be little risk, however, of inducing this storage disorder when using the drug in a dose of 50 mg *per os* for treatment of type II diabetes. In high doses, the drug may prove useful for studying the pathogenesis of glycogenosis type II *in-vitro* or in animal models.

Introduction

The glucose analogue N-hydroxyethyl-1-deoxynojirimycin (BAY m 1099; miglitol) is a potent inhibitor of intestinal α -glucosidases and indirectly limits the absorption of monosaccharides in the gut (1,2). Other derivatives of nojirimycin like 1-deoxynojirimycin, N-methyl-1-deoxynojirimycin and BAY o 1248, and the pseudotetrasaccharide acarbose have a similar effect. Some of these compounds have been used in the past few years in clinical trials for treatment of type II diabetes and appeared to be effective in reducing the postprandial rise of the blood glucose level (2-5).

¹Adapted from Clin. Chim. Acta 182: 41-52 (1989).

Other than for clinical application these drugs are used as a tool for examining the effect of carbohydrate chain modifications on the biosynthesis and intracellular transport of glycoproteins. These proteins acquire N-linked oligosaccharide chains in the lumen of the rough endoplasmic reticulum (see ref. 6 for review). The first three glucose residues of the original $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ oligosaccharide structure are removed by α -glucosidase I and II, whereafter the carbohydrate composition is further modified by the sequential action of glycosidases and glycosyltransferases. This processing occurs while the protein is in transit through the endoplasmic reticulum and the Golgi cisternae. The glucose derivatives 1-deoxynojirimycin and N-methyl-1-deoxynojirimycin, and other compounds like bromoconduritol and castanospermine are known inhibitors of glucosidases I and/or II and induce accumulation of glycoproteins in the endoplasmic reticulum (7-11).

Furthermore, Lüllmann-Rauch (12) and Chambers et al. (13) have shown that acarbose and nojirimycin are also inhibitors of lysosomal α -glucosidase. Thus, intraperitoneal injections of acarbose in rats (12) induce intralysosomal glycogen storage in liver and other organs. The experimentally induced alterations resemble those characteristically observed in the inherited disease glycogenosis type II (Pompe's disease). In the present study we have examined the effect of BAY m 1099 on the biosynthesis and function of lysosomal α -glucosidase in human skin fibroblasts and HepG2 cells.

Results

Figure 1 shows that BAY m 1099 is a potent inhibitor of lysosomal α -glucosidase. Using purified placental enzyme as "target" and 4-methylumbelliferyl- α -D-glucopyranoside as substrate the mode of action of BAY m 1099 was characterized as a noncompetitive type of inhibition (Lineweaver-Burk plot, Fig. 1A). With glycogen as substrate, BAY m 1099 showed a competitive type of inhibition (Fig. 1B). The inhibitor constants were graphically determined from Dixon plots (not shown). The k_i value with 4-MU as substrate was 2.75×10^{-6} M, while the k_i with glycogen was 1.25×10^{-6} M.

To determine whether the inhibitor would also affect α -glucosidases *in vivo*, fibroblasts and HepG2 cells were exposed for three days to different concentrations of BAY m 1099. After this period the activity of lysosomal acid α -glucosidase in fibroblasts was 70% reduced (Fig. 2A), while the acid α -glucosidase activity in HepG2 cells was inhibited for about 80% at the highest concentration of inhibitor tested (4 mM; Fig. 2B). In other experiments with HepG2 cells a maximal inhibition of 85-90% was observed with 10 mM of inhibitor. The neutral α -glucosidase activity in fibroblasts was decreased for about 33% after treatment with inhibitor (Fig. 2A). In HepG2 cells the highest concentration of inhibitor tested (4 mM) resulted in a 50% decrease of neutral α -glucosidase activity (Fig. 2B).

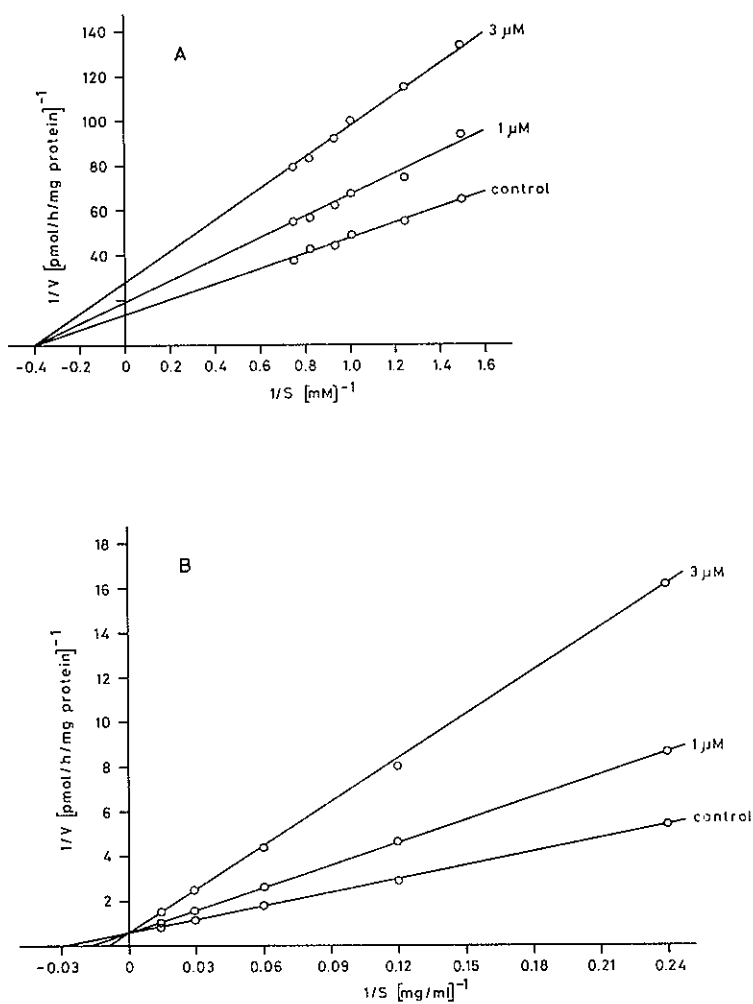


Figure 1. Kinetic analysis of placental acid α -glucosidase inhibition by BAY m 1099 (Lineweaver-Burk plot). The inhibitor was used in final concentrations of 1 and 3 μ M as indicated.
A: Inhibition of 4-methylumbelliferyl- α -D-glucopyranoside hydrolysis.
B: Inhibition of glycogen hydrolysis.

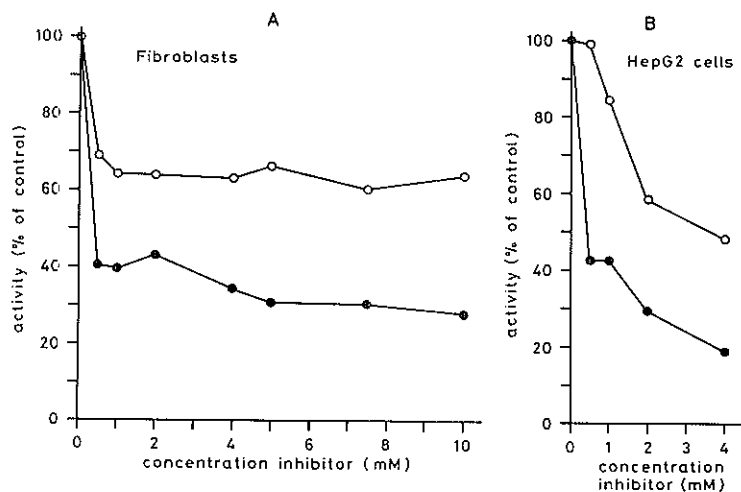


Figure 2. Effect of BAY m 1099 on acid (●) and neutral (○) α -glucosidase activity in cultured fibroblasts (A) and HepG2 cells (B). The acid α -glucosidase activity of fibroblasts and HepG2 cells in the absence of inhibitor was 71.93 and 163.8 nmol/h per mg of protein, respectively. The neutral α -glucosidase activity of these cells in the absence of inhibitor was 29.8 and 61.8 nmol/h per mg of protein, respectively. Assays were as described in 'materials and methods' using 4-methylumbelliferyl- α -D-glucopyranoside as substrate.

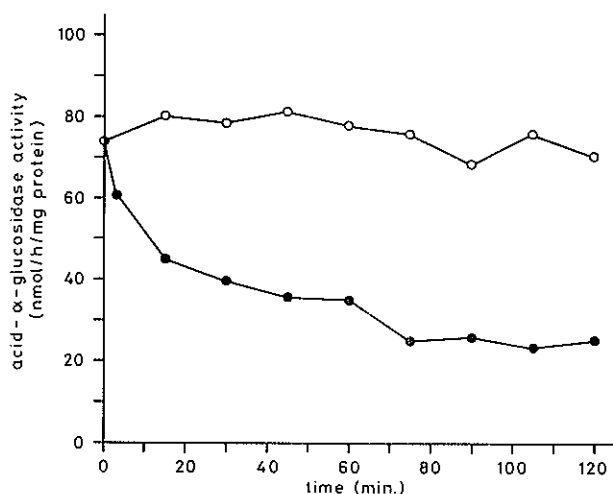


Figure 3. Time-dependent inhibition of acid α -glucosidase activity by BAY m 1099. At $t=0$, 4 mM inhibitor was added to the culture medium (●). Control cultures obtained fresh medium without inhibitor (○).

Figure 3 shows that the inhibition of the acid α -glucosidase activity of HepG2 cells reaches a maximum within 2 h. A similar time-dependent inhibition of neutral α -glucosidase activities was observed (data not shown). In the experiment depicted in Fig. 4 it was determined whether the inhibition of neutral and acid α -glucosidases was reversible. Fibroblasts and HepG2 cells were cultured for 7 days in the presence of 10 mM inhibitor (the culture medium was refreshed each 2 days). At day 7, half of the cultures received fresh medium without inhibitor, whereas the other half was kept in medium with inhibitor for another 2 days. During these days, cells were collected and the enzyme activity was measured (Fig. 4A,B). After the first seven days with inhibitor the residual activity of acid and neutral α -glucosidases of HepG2 cells was 15% and 28%, respectively. One day after removal of the inhibitor the acid α -glucosidase activity had increased to 60%, and within 48 h the control level was almost reached (Fig. 4A). A similar response was found for neutral α -glucosidase activity. Within 24 h the activity had returned to 87% of control level and had reached 100% after 48 h (Fig. 4B). Inhibition of *de novo* protein synthesis with cycloheximide (10 μ g/ml) had no effect on the return of acid and neutral α -glucosidase activity (data not shown).

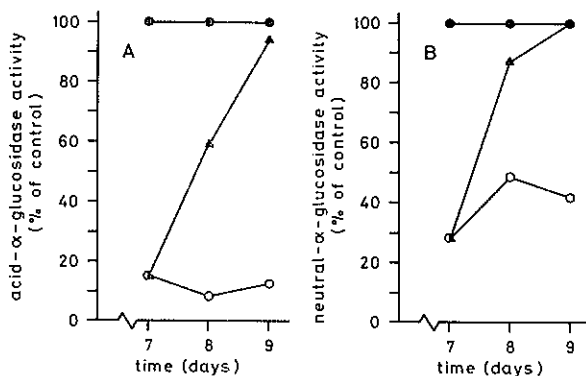


Figure 4. Inhibition and recovery of α -glucosidase activities in BAY m 1099 treated HepG2 cells. Cells were cultured for 7 days in medium with 10 mM BAY m 1099. After this period the acid (A) and neutral α -glucosidase (B) activities were measured during the following two days in medium with (○) or without (●) inhibitor. Control cells were maintained in medium without inhibitor during the whole period (●).

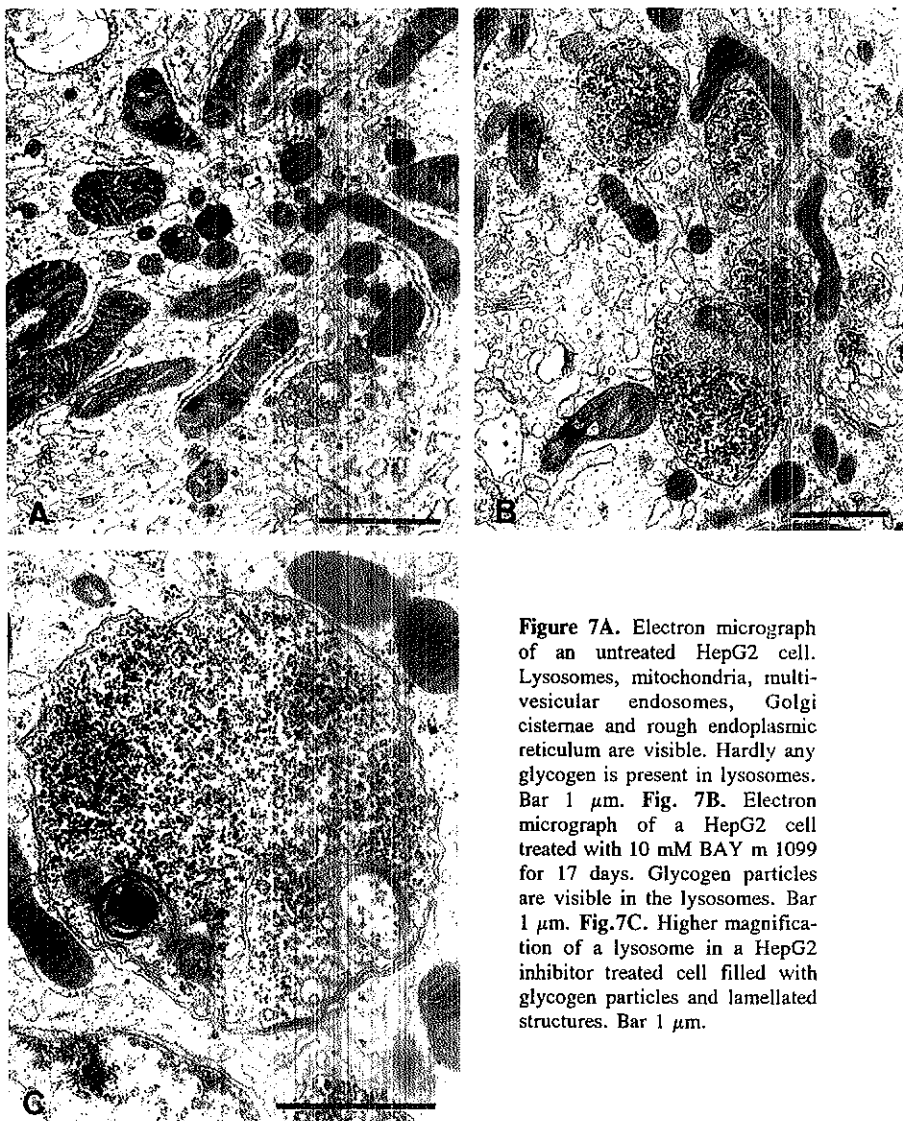


Figure 7A. Electron micrograph of an untreated HepG2 cell. Lysosomes, mitochondria, multi-vesicular endosomes, Golgi cisternae and rough endoplasmic reticulum are visible. Hardly any glycogen is present in lysosomes. Bar 1 μ m. **Fig. 7B.** Electron micrograph of a HepG2 cell treated with 10 mM BAY m 1099 for 17 days. Glycogen particles are visible in the lysosomes. Bar 1 μ m. **Fig. 7C.** Higher magnification of a lysosome in a HepG2 inhibitor treated cell filled with glycogen particles and lamellated structures. Bar 1 μ m.

To study the effect of BAY m 1099 on acid α -glucosidase synthesis, fibroblasts and HepG2 cells were labelled with [^3H]-leucine for 20 h in the presence of 4 mM inhibitor. The molecular forms of acid α -glucosidase synthesized in the absence or presence of inhibitor are shown in Fig. 5. The enzyme is synthesized as a precursor with an apparent molecular mass of 110 kDa which is processed via a 95 kDa intermediate to a mature species of 76 kDa (17). In the presence of inhibitor accumulation of the 95 kDa processing intermediate was observed (Fig. 5), and the amount of 76 kDa mature enzyme was reduced. The molecular mass of the different acid α -glucosidase species appeared not to be changed in the presence of inhibitor.

In view of the fact that the inhibitor delays processing of newly formed lysosomal α -glucosidase and affects the activity of mature enzyme, lysosomal glycogen storage may be anticipated. To investigate this, electron microscopy was performed. Fig. 6A and B presents ultrathin frozen sections of inhibitor treated and untreated HepG2 cells in which acid α -glucosidase is localized by immunocytochemistry. The enzyme is present in lysosomes (Fig. 6A,B), rough endoplasmic reticulum, throughout the stacks of Golgi cisternae, along the plasmamembrane and in multivesicular endosomes. Similar results were obtained with human fibroblasts (not shown). There was no clear difference between treated and untreated cells. However, in thin epon sections of inhibitor treated HepG2 cells morphological differences were observed in comparison with untreated cells (Fig. 7A,B). In inhibitor treated cells, glycogen had accumulated in most lysosomes. Figure 7C shows a higher magnification of lysosomes filled with glycogen particles. Similar glycogen loaded lysosomes were observed in human fibroblasts (not shown).

Discussion

BAY m 1099 (miglitol) is currently tested in the clinic for treatment of type II diabetes. When administered orally in proper dose, the drug inhibits intestinal sucrase and maltase activity and prevents rise of the blood glucose level after ingestion of sucrose, maltose and starch (2,4,5). BAY m 1099 is a more potent inhibitor than acarbose which has a similar effect (1). The drug is tolerated well and does not seem to elicit short-term side effects. In contrast to acarbose, however, BAY m 1099 is absorbed in the intestine with the potential risk of disturbing cellular metabolism (4). For this reason we have investigated the effect of BAY m 1099 on the activity of intracellular neutral and acid α -glucosidases.

Our data show this glucose analogue to be a potent inhibitor of purified human placental acid α -glucosidase. Strong inhibition of enzyme activity towards both the natural substrate glycogen and the artificial substrate 4-methylumbelliferyl- α -D-glucopyranoside is measured. Glycogen hydrolysis is inhibited in a competitive manner whereas the artificial substrate is inhibited non-competitively. Comparison of BAY m 1099 and nojirimycin (13)

reveals that their mode of inhibition is different, because nojirimycin inhibits the hydrolysis of 4-MU and glycogen in an uncompetitive way. Modification of nojirimycin apparently alters its binding characteristics. The inhibitor constants of BAY m 1099 and nojirimycin are similar, and in the micro-molar range. Lembcke et al. (1) studied the inhibition kinetics of BAY m 1099 towards rat small intestinal sucrase and found a competitive type of inhibition. The inhibition constant (k_i) was reported to be 1.14×10^{-7} M, indicating that BAY m 1099 has a 440,000-fold higher affinity for sucrase than sucrose. Our calculations reveal that BAY m 1099 has a 130,000-fold higher affinity for placental acid α -glucosidase than glycogen. Compared to 4-methylumbelliferyl- α -D-glucopyranoside the affinity is 900 fold higher. The analogous response of lysosomal α -glucosidase and sucrase to BAY m 1099 is not fully unexpected in view of the fact that the primary structure of these two enzymes around their presumed active sites is rather similar (26).

The experiments with cultured fibroblasts and HepG2 cells show that BAY m 1099 also exerts an effect on the activity of lysosomal and neutral α -glucosidases *in situ*. The lysosomal α -glucosidase is inhibited for 70-90% but only when the nojirimycin derivative is added to the culture medium in a concentration above 4 mM. Neutral α -glucosidase activity was inhibitable to a maximum of 70% at 10 mM inhibitor. Since several α -glucosidases have been identified with a neutral pH optimum it is unclear whether different enzymes are inhibited to the same extent or whether some neutral α -glucosidases are fully inactivated while others are unaffected. But the observed reduced rate of lysosomal enzyme maturation suggests that BAY m 1099 has an effect on the neutral carbohydrate processing enzymes α -glucosidase I and II. Early processing intermediates of lysosomal α -glucosidase (95 kDa) appear to accumulate in the presence of inhibitor while there is little formation of 76 mature enzyme. A similar delay in the processing of glycoproteins has also been reported by Lemansky et al. (8) and Nauerth et al. (9) using 1-deoxynojirimycin and Arakaki et al. (11) using castanospermine as inhibitors of α -glucosidase I and II. In contrast to these studies, however, we did not detect enzyme precursors with abnormal molecular mass.

The accumulation of the 95 kDa form of acid α -glucosidase by BAY m 1099 did not result in a visibly abnormal localization of the enzyme in any of the intracellular compartments involved in lysosomal enzyme transport. Nevertheless, a long term effect of BAY m 1099 on lysosomal enzyme biosynthesis can not be excluded. The almost instant loss of lysosomal α -glucosidase activity upon addition of BAY m 1099 must be caused by direct inhibition of mature enzyme in the lysosomes. The rapid reversibility of enzyme inhibition after removal of the inhibitor in the absence of protein synthesis supports this view.

It is this direct effect of BAY m 1099 on lysosomal α -glucosidase which deserves particular attention. The residual activity of acid α -glucosidase in fibroblasts and HepG2 cells in the presence of 10 mM inhibitor varied from 10-30%. This level of activity is typically measured in cells from patients with an adult form of the lysosomal glycogen storage

disorder glycogenosis type II (17,27). Indeed, our electronmicroscopic studies reveal that accumulation of lysosomal glycogen is induced when cells are exposed for longer periods to the relatively high concentration of BAY m 1099. The oral dose (50 mg) given to patients with type II diabetes is not likely to lead to glycogenosis since the inhibitor concentration is far too low (approximately $3 \mu\text{mol/l}$) when the drug is fully absorbed and equally distributed over the whole body. But, the concentration of the drug in intestinal epithelial cells and the underlying tissue may be higher. When administered to animals in sufficiently high dose, BAY m 1099 and other α -glucosidase inhibitors like acarbose (12,28) and castanospermine (29) will be useful for studying the cytological changes and pathological phenomena which occur in glycogenosis type II.

Acknowledgements

N-hydroxyethyl-1-deoxynojirimycin BAY m 1099/miglitol was generously supplied by Dr. J.H. Branolte (Bayer Nederland B.V.). Advice on the use of BAY m 1099 was obtained from Dr. F. Seuter (Bayer AG, Wuppertal). We are grateful to R. Willemsen and W.J. Visser for technical assistance, J. Fengler for preparing the illustrations and D. Heinsius and J. Lokker for secretarial assistance. Financial support was obtained from the Netherlands organization for scientific research (N.W.O.).

Materials and methods

Cells

Human skin fibroblasts from healthy individuals were obtained from the Rotterdam Cell Repository (Prof. Dr. M.F. Niermeijer). The human hepatoma cell line, HepG2, used in this study is described by Knowles (14). Cells were maintained at 37°C under 10% CO_2 in Dulbecco's modified Eagle's medium (Flow laboratories Inc. McLean, VA) supplemented with 10% fetal calf serum and antibiotics (100 μg of streptomycin and 100 U penicillin/ml; Gibco). Cells at exponential phase of growth were used for all studies. N-hydroxyethyl-1-deoxynojirimycin (BAY m 1099, kindly provided by Dr. J.H. Branolte; Bayer Nederland B.V.) was added to the culture medium in concentrations as mentioned in 'Results'. For metabolic labelling, cells (grown in 25 cm^2 tissue culture flasks; Costar) were preincubated with the inhibitor for 24 h prior to labelling. The inhibitor was present throughout the labelling procedure.

Biochemical assays

Protein concentrations were measured by the method of Lowry et al. (15), using bovine serum albumin as standard. Acid α -glucosidase (EC 3.2.1.3) activity was determined with the artificial substrate 4-methylumbelliferyl- α -D-glucopyranoside (Koch Light Chemical Companies) as described before (16). Neutral α -glucosidase activity was measured by incubating 10 μl cell homogenate with 20 μl of 2.2 mM 4-methylumbelliferyl- α -D-glucopyranoside (4-MU) in 0.05 M potassiumphosphate buffer, pH 7.0, for 1 h at 37°C . Kinetic analysis of the inhibition of placental acid α -glucosidase by BAY m 1099 was carried out with the artificial 4-MU substrate and with the natural substrate glycogen. The inhibitor was used at final concentrations of 1 and 3 μM . The 4-MU substrate concentrations used were 0.67/0.80/0.93/1.07/1.20 and 1.33 mM. All assays utilizing 4-MU as substrate contained 5 ng of purified human placental acid α -glucosidase (17). Glycogen was used in final concentrations of 4.2/8.3/16.6/33.3 and 66.6 mg/ml. All assays with glycogen contained 15 ng of purified human placental acid α -glucosidase. Glucose liberation was measured by the glucose oxidase method (18).

Metabolic labelling

For labelling studies fibroblasts and HepG2 cells were preincubated in leucine free medium with 2% dialyzed fetal calf serum for 60 min. Labelling was performed in the same medium to which L-(4,5 ^3H) leucine (0.04 mCi/ml) was added (Amersham Radiochemical Centre, England). After 20 h, cells were harvested and

solubilized as described by Hasilik and Neufeld (19). Acid α -glucosidase was immunoprecipitated with affinity purified antibodies against human placental acid α -glucosidase (20). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed on 10% slab gels according to Laemmli (21). The radioactive bands were visualized by fluorography.

Electron microscopy

Fibroblasts and HepG2 cells were treated with 10 mM BAY m 1099 for a period of 14 and 17 days, respectively. The medium was refreshed each 3 days. Cells were fixed in 3% glutaraldehyde in 0.1 M cacodylate-buffer, pH 7.3, for 4.5 h, at 4°C. After rinsing for 2 h at 4°C in the same buffer, the cells were postfixed for 16 h at 4°C in 1% OsO₄ in 0.1 M cacodylate-buffer, pH 7.15, plus 50 mM K₃Fe(CN)₆ for visualization of glycogen as described by De Bruyn (22). After rinsing in 0.1 M cacodylate-buffer, pH 7.3, at room temp. the cells were dehydrated in alcohol and embedded in epon. Ultrathin sections were cut with a Reichert OMU3 ultramicrotome. These sections were stained with uranyl acetate and lead citrate and viewed in a Philips EM400 electronmicroscope at 80 kV.

Immunocytochemistry

Fibroblasts and HepG2 cells were grown in 75 cm² tissue culture flasks (Costar). After treatment for one week with 10 mM inhibitor, cells were harvested and prepared for immunocytochemistry as described before (23). Ultracryotomy was carried out as described by Tokuyasu (24) using a LKB Nova ultratome equipped with the Cryo Nova. The methods used for immuno electronmicroscopy were those described before (23). Briefly, affinity purified antibodies against human acid α -glucosidase (20) were used for the first incubation step. Antigen-antibody complexes were visualized by using goat anti-(rabbit IgG) coupled to 10 nm colloidal gold (GAR-10, Janssen Pharmaceutica Beersse, Belgium). Sections were stained with uranyl acetate and embedded in 1.5% methylcellulose (25). Control sections were treated only with goat anti-(rabbit IgG) coupled to 10 nm colloidal gold. Background labelling was negligible.

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Chapter 6

Human Lysosomal α -Glucosidase: Functional Characterization of the Glycosylation Sites¹

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Summary

N-linked glycosylation is one of the important events in the post translational modification of human lysosomal α -glucosidase. Phosphorylation of mannose residues ensures efficient transport of the enzyme to the lysosomes via the mannose 6-phosphate receptor. The primary structure of lysosomal α -glucosidase, as deduced from the cDNA sequence, indicates that there are seven potential glycosylation sites. We have eliminated these sites individually by site directed mutagenesis and thereby demonstrated that all seven sites are glycosylated. The sites at Asn 882 and Asn 925 were found to be located in a C-terminal pro-peptide which is cleaved off during maturation. Evidence is presented that at least two of the oligosaccharide side chains of human lysosomal α -glucosidase are phosphorylated. Elimination of six of the seven sites does not disturb enzyme synthesis or function. However removal of the second glycosylation site at Asn 233 interferes dramatically with the formation of mature enzyme. The mutant precursor is synthesized normally and assembles in the endoplasmic reticulum, but immuno-electron microscopy reveals a deficiency of α -glucosidase in the Golgi complex and in the more distal compartments of the lysosomal transport pathway.

Introduction

Lysosomal α -glucosidase (EC 3.2.1.3) is a glycoprotein like the other acid hydrolases that perform their function in the lysosomes. The function of α -glucosidase is degradation of glycogen to glucose. Enzyme deficiency leads to glycogenosis type II, an inherited glycogen storage disorder (Hers 1963). Several patients have been described with a defect

¹Adapted from Biochem J. 289: 681-686 (1993).

in the synthesis or post translational processing of lysosomal α -glucosidase (Reuser et al. 1982, 1985, 1987, Beratis et al. 1983, Martiniuk et al. 1986, 1990a, Van der Ploeg et al. 1989, Hermans et al. 1991, Zhong et al. 1991).

During translation, lysosomal enzymes enter the endoplasmic reticulum where glycosylation is assumed to start even before the protein is completely folded (Rothman et al. 1978). Asparagine residues in the sequence Asn-Xaa-Ser/Thr (Xaa all but Pro) are the potential sites for attachment of N-linked carbohydrate side chains which are transferred *en block* from dolichol pyrophosphate (Marshall 1972, Kornfeld and Kornfeld 1985). However, not all potential glycosylation sites are used. Studies by Bause and Legler (1981) indicate that a proper protein conformation is required for recognition and glycosylation of a site. After removal of the three terminal glucose residues of the oligosaccharide precursor chain in the endoplasmic reticulum (Kornfeld et al. 1978, Hubbard and Robbins 1979) most lysosomal enzymes obtain a mannose 6-phosphate recognition marker as lysosomal targeting signal (Creek and Sly 1984). This is accomplished by the transfer of N-acetylglucosamine 1-phosphate from UDP-N-acetylglucosamine to particular mannose residues (Reitman and Kornfeld 1981, Waheed et al. 1981), and the subsequent uncovering of the phosphate by a phosphodiesterase which is probably localized in the mid-Golgi (Lazzarino and Gabel 1988). Transport to the lysosomes continues via the trans Golgi cisternae to the trans Golgi reticulum. Binding to the mannose 6-phosphate receptor in this part of the transport pathway is essential for lysosomal targeting. Lysosomal enzymes are then transported to the late endosomes (Griffiths and Simons 1986), where a fall in pH causes the ligand to dissociate from the receptor. The enzymes continue their way to the lysosomes and the receptor cycles back to the Golgi complex (Brown et al. 1986, Von Figura and Hasilik 1986).

Lysosomal α -glucosidase is known to follow this transport route and is subject to several post translational modifications involving both the carbohydrate chains and the protein backbone. The enzyme is synthesized as a glycosylated precursor of approximately 110 kD, which is phosphorylated. The amino acid sequence of lysosomal α -glucosidase, as it was derived from the cloned cDNA, indicates that there are seven potential glycosylation sites (Hoefsloot et al. 1988, Martiniuk et al. 1990b). Proteolytic processing gives rise to a 95 kD intermediate form and results finally in the formation of two lysosomal enzyme species of 76 kD and 70 kD (Hasilik and Neufeld 1980, Reuser et al. 1985). The latter two forms of lysosomal α -glucosidase have been purified and they were analyzed with respect to their sugar content and carbohydrate chain structure. It was estimated that lysosomal α -glucosidase from human placenta and liver contains an average of four to five carbohydrate chains (Belenky et al. 1979, Mutsaers et al. 1987).

The aim of this study was to establish the actual number of glycosylation sites and to determine the role of each individual site in transport and catalytic function of lysosomal α -glucosidase. The approach that was taken was to eliminate the potential sites by site directed

mutagenesis and to study the effect by expression of the mutant cDNA constructs *in vitro* and in transiently transfected COS cells.

Results

In lysosomal α -glucosidase asparagine residues in the recognition sequence for N-linked glycosylation Asn-Xaa-Thr/Ser (Xaa all but Pro) are found at seven positions, namely at Asn 140, 233, 390, 470, 652, 882 and 925 (Hoefsloot et al. 1988, Martiniuk et al. 1990b)(EMBL entry number Y00839). These potential glycosylation sites were eliminated one by one to determine which sites were actually used, and to examine the importance of individual sites for enzyme function and lysosomal targeting. To this end the recognition consensus sequence of each potential site was altered by site directed mutagenesis of the lysosomal α -glucosidase cDNA inserted in M13mp19. The codon for Asn was replaced by one coding for the very similar amino acid residue Gln, using the oligonucleotides listed in Table II (see materials and methods). The mutants, each missing a different glycosylation site, were designated Δ G1 - Δ G7 in order of appearance from the N-terminal end.

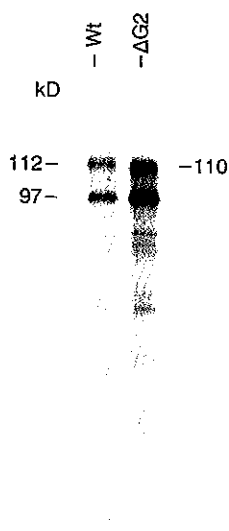


Figure 1. In vitro translation of human lysosomal α -glucosidase. Wild type and mutant (Δ G2) cDNA's were transcribed *in vitro* and the mRNA's were translated in a rabbit reticulocyte lysate in the presence of canine pancreatic microsomes. The proteins were labelled with [35 S]-methionine, immunoprecipitated, and separated by SDS-PAGE. The molecular mass of the unglycosylated and glycosylated lysosomal α -glucosidase precursors is indicated.

In vitro transcription and translation.

The use of glycosylation sites was tested by cloning the wild type and mutant cDNA's in the eukaryotic expression vector pSG5 allowing *in vitro* transcription and subsequent translation of the mRNA's in the presence of dog pancreatic microsomes. Translation of wild type mRNA resulted in the formation of two molecular species, a translocated and glycosylated precursor of 112 kD and an unglycosylated precursor of 97 kD (Figure 1, and Van der Horst et al. 1987). The glycosylation sites are easily identified by this method. Figure 1, for instance, shows that the substitution of Asn 233 by Gln 233 ($\Delta G2$) results in a 2 kD size reduction of the glycosylated precursor, whereas the apparent size of the unglycosylated wild type and mutant precursor remain the same (97 kD).

Transient expression in COS cells.

The functional consequence of the removal of glycosylation sites was investigated by following the synthesis and maturation of α -glucosidase in transiently transfected COS cells. [3 H]-leucine labelled wild type and mutant lysosomal α -glucosidase species were immunoprecipitated from cell homogenates after a 2 h pulse period or after a subsequent chase of 16 h, and analyzed by SDS-PAGE (Figure 2). The secreted form of lysosomal α -glucosidase was immunoprecipitated from the culture media and analyzed by Western blotting (Figure 3). In cells transfected with wild type cDNA the lysosomal α -glucosidase precursor of 110 kD is synthesized (pulse) and converted to a 95 kD intermediate and a 76 kD mature species (chase) (Figure 2). The culture medium contains only the secreted 110 kD precursor (Figure 3). All mutant precursor proteins (isolated from cells and media) missing one potential glycosylation site appeared to have a slightly lower molecular mass (108 kD) than the wild type precursor. This indicates that all seven potential glycosylation sites of lysosomal α -glucosidase are used. For the mutants lacking the first, third, fourth or fifth glycosylation site ($\Delta G1$, $\Delta G3$, $\Delta G4$ and $\Delta G5$) the lower molecular mass is maintained during the maturation process leading to the formation of a 93 kD processing intermediate and a 74 kD mature enzyme species (Figure 2, chase). A different effect was observed with the mutants $\Delta G6$ and $\Delta G7$. The precursor and the processing intermediate were smaller (93 kD) than the comparable wild type species, but the final maturation products of $\Delta G6$ and $\Delta G7$ (76 kD) and wild type lysosomal α -glucosidase were of the same size (Figure 2, chase). This indicates that a C-terminal peptide containing the glycosylation sites at Asn 882 and Asn 925 is cleaved off when lysosomal α -glucosidase matures from 95 to 76 kD.

The only glycosylation site which has a severe effect on the biosynthesis of α -glucosidase when eliminated is Asn 233 (Figure 2). The mutant precursor of 108 kD is evidently formed during the 2 h pulse, but the 93 kD processing intermediate and the 74 kD mature enzyme are clearly deficient in the chase. The $\Delta G2$ precursor is not secreted into the culture medium in contrast with all other mutant precursors which are secreted normally (Figure 3).

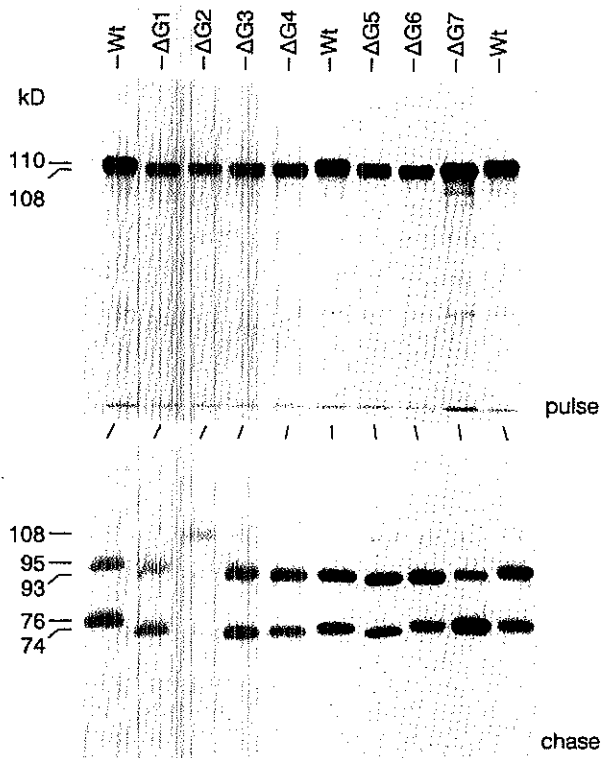


Figure 2. Synthesis and processing of wild type and mutant lysosomal α -glucosidase in COS cells. Cos cells transfected with wild type and mutant cDNA constructs were labelled for 2 h with [3 H]-leucine and harvested directly (pulse) or after a subsequent period of 16 h (chase). Lysosomal α -glucosidase was immunoprecipitated and analyzed by SDS-PAGE. The molecular mass of wild type and mutant lysosomal α -glucosidase species is indicated.

To investigate the effect of glycosylation on the catalytic activity of lysosomal α -glucosidase, cell homogenates and culture media of transfected COS cells were assayed for enzyme activity. COS cells transfected with *E. coli* β -galactosidase cDNA were taken as a reference. The data of a typical transfection experiment and the average outcome of the different experiments are given in Table I. When compared to wild type, all glycosylation site defective mutants except Δ G2 exhibited intracellularly a similar activity of lysosomal α -glucosidase. Δ G2 was different in that only 5% of wild type activity was measured in the cell homogenates and less than 3% in the medium. Near normal levels of lysosomal α -glucosidase were secreted by COS cells transfected with the other mutant constructs (Table I).

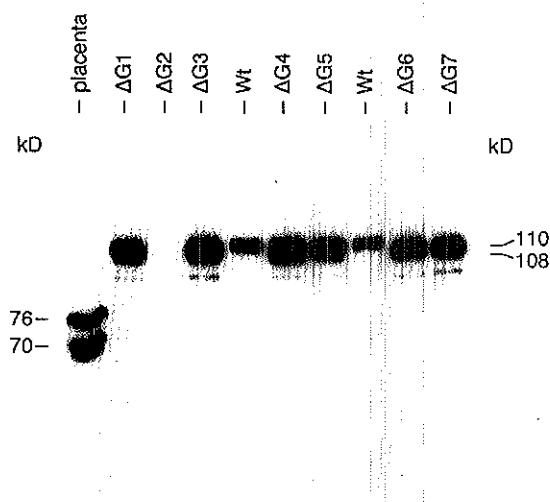


Figure 3. Secretion of wild type and mutant lysosomal α -glucosidase by transiently transfected COS cells. Lysosomal α -glucosidase was immunoprecipitated from the culture media at 90 h after transfection and analysed by Western blotting after SDS-PAGE. The molecular mass of wild type and mutant lysosomal α -glucosidase species is indicated.

Phosphorylation of lysosomal α -glucosidase

To determine which of the seven oligosaccharide chains of lysosomal α -glucosidase were phosphorylated, we transfected COS-cells with the wild type and mutant cDNA constructs and labelled the cells with [32 P] P_i . After a 6 h labelling period phosphorylated precursor and intermediate forms of lysosomal α -glucosidase were observed in cells expressing either wild type or mutant enzyme (Figure 4). The mature forms of lysosomal α -glucosidase became detectable after a subsequent chase of 12 h, except in cells expressing Δ G2. The [32 P] P_i labelling procedure appeared sensitive enough to demonstrate conversion of the Δ G2 precursor to the 93 kD intermediate but the formation of mature (74 kD) enzyme remained undetectable. When [32 P] P_i labelled wild type lysosomal α -glucosidase was incubated with endoglycosidase F, prior to SDS-PAGE, no phosphorylated protein could be detected, indicating that the [32 P] P_i was linked to the mannose residues of the carbohydrate side chains (results not shown).

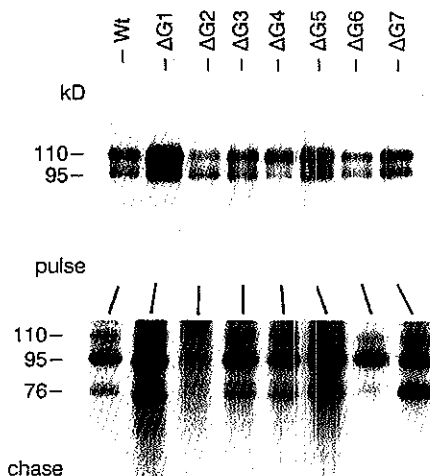
Table I

Activity of the glycosylation mutants of lysosomal α -glucosidase in transiently transfected COS cells measured 90 h after transfection.

TYPE OF MUTATION	CATALYTIC ACTIVITY*			
	CELLS		MEDIUM	
wild type	960	(100)	406	(100)
Δ G1	665	(61.7 \pm 7.4)	175	(42.6 \pm 5.5)
Δ G2	116	(5.0 \pm 2.3)	15	(2.1 \pm 0.9)
Δ G3	789	(77.9 \pm 9.1)	307	(89.2 \pm 15.7)
Δ G4	671	(69.8)	275	(67.4)
Δ G5	645	(66.8 \pm 6.6)	152	(89.1 \pm 30.1)
Δ G6	919	(90.1 \pm 7.7)	304	(75.9 \pm 5.2)
Δ G7	1027	(109.2 \pm 6.9)	473	(116.6 \pm 0.1)
β -Gal construct	50	(0)	3	(0)

* The activity is expressed as nmol MU/mg protein/h. The activities compared to wild type are given within brackets as percentage \pm SEM (n=1-4).

Figure 4. Phosphorylation of wild type and mutant lysosomal α -glucosidase in COS cells. COS cells transfected with wild type and mutant cDNA constructs were labelled for 6 h with [32 P]P_i and harvested directly (pulse) or after a subsequent period of 12 h (chase). Lysosomal α -glucosidase was immunoprecipitated from the cell homogenates and separated by SDS-PAGE (10% acrylamide, 1% crosslink). The molecular mass of the wild type lysosomal α -glucosidase species is indicated.



Intracellular transport

The intracellular localization of the mutant α -glucosidase species missing one glycosylation site was investigated using immunocytochemistry. As observed in earlier studies, the formation of mature enzyme was in all instances correlated with a typical punctate lysosomal labelling pattern. Figure 5a, for instance, illustrates the lysosomal localization of Δ G4. Transfection of COS cells with Δ G2, showing an apparent maturation defect, resulted in a diffuse network of labelled structures spreading from the nucleus into the cytoplasm (Figure 5b). The exact intracellular localization of Δ G2 was revealed by immuno-electron microscopy (Figure 6). Labelling of the nuclear envelope and the ER was obtained but the enzyme could not be detected in the Golgi complex nor in the TGR or the lysosomes. The later compartments were labelled after transfection with the wild type cDNA construct (results not shown).

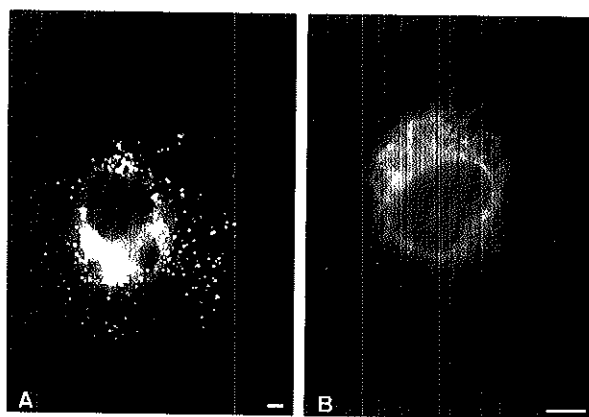


Figure 5. The localization of glycosylation site deficient lysosomal α -glucosidases in transfected COS cells studied by light-microscopy.

Transiently transfected cells were fixed and incubated with a rabbit polyclonal antiserum against human lysosomal α -glucosidase. Immune complexes were visualized with goat anti-(rabbit IgG) conjugated to fluorescein. A: Δ G4. B: Δ G2. Bar represents 10 μ m.

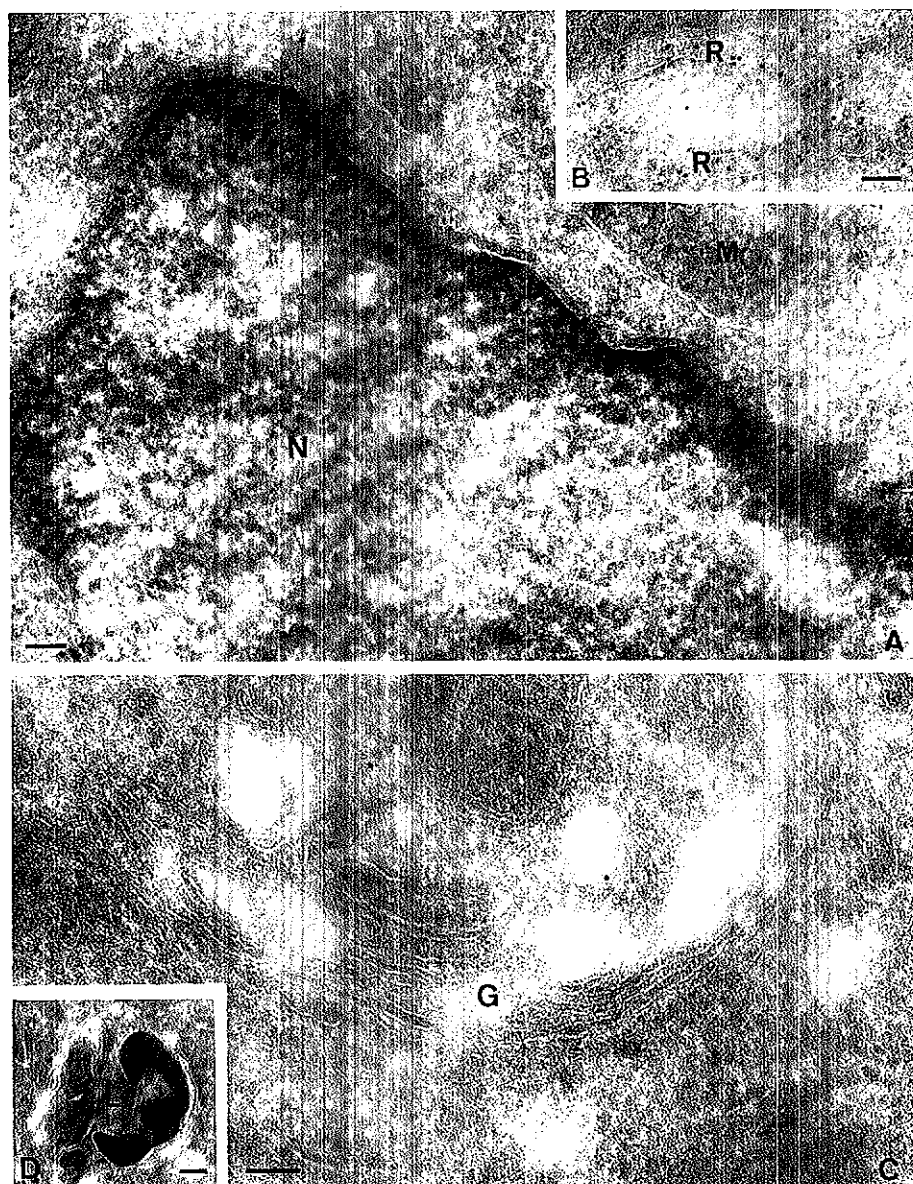


Figure 6. The intracellular localization of $\Delta G2$ in transfected COS cells studied by immuno electron microscopy. Ultra-thin cryosections were incubated with rabbit polyclonal antiserum against human lysosomal α -glucosidase and subsequently with goat anti-(rabbit IgG) coupled to colloidal gold. Mutant lysosomal α -glucosidase was found at the nuclear envelop (A) and in the endoplasmic reticulum (B), but not in the Golgi complex (C), or the lysosomes (D). N: nucleus, M: mitochondrion, R: rough endoplasmic reticulum, G: Golgi complex, L: lysosomes. In all figures bar represents 0.1 μm .

Discussion

Site directed mutagenesis was applied to determine the glycosylation sites of lysosomal α -glucosidase and to define their significance for enzyme function and lysosomal targeting. The removal of one high mannose type of carbohydrate chain is expected to result in a molecular mass reduction of approximately 2 kD and to lead to a comparable increase in electrophoretic mobility on SDS-PAGE. This effect was observed upon deletion of each of the seven potential glycosylation sites of the 110 kD precursor. The mobility shift in the more mature forms of α -glucosidase is informative with respect to the sites of proteolytic processing. The 2 kD size reduction is maintained after formation of the 95 kD processing intermediate irrespective of the deleted glycosylation site. This implies that the peptides that are cleaved off during the conversion of 110 kD to 95 kD do not contain a glycosylation site. Since the seventh glycosylation site is located just thirty-one residues from the C-terminal end of the 110 kD precursor (Hoefsloot et al. 1988) we conclude that the proteolytic conversion occurs mainly at the N-terminal end of the precursor. Upon further maturation of the enzyme to 76 kD the mutants lacking the sixth and the seventh glycosylation site at Asn 882 and Asn 925 are no longer smaller than the wild type species whereas the other mutants are still reduced in size. Thus, the maturation from 95 to 76 kD involves C-terminal processing at a site upstream of Asn 882. Based on the N-terminal sequence of the 76 kD form of lysosomal α -glucosidase and the sequence of C-terminal tryptic peptides (Hoefsloot et al. 1988) we estimate that the site is located between amino acid residue 820 and 880.

According to the amino acid sequence of lysosomal α -glucosidase the first glycosylation site is located in between the N-terminal ends of the 76 and 70 kD species purified from human placenta. This leaves four sites occupied in the 70 kD species. These findings are in line with the earlier estimates of Mutsaers et al. (1987) on the number of carbohydrate chains attached to human placental lysosomal α -glucosidase. A figure of four to five side chains per polypeptide molecule was obtained for a preparation containing equimolar amounts of the 76 and 70 kD species.

Six of the seven mutants, each lacking a different glycosylation site, are transported to the lysosomes and are catalytically active. Also the proteolytic processing and the secretion of these mutants is normal. But elimination of the second glycosylation site at Asn 233 has a dramatic effect. The precursor (108 kD) is normally synthesized but the intermediate and mature forms of α -glucosidase are severely reduced in amount. The relative persistence of the Δ G2 precursor during the 16 h chase (Figure 2) suggests a delayed transport of the mutant precursor from the ER to the Golgi. This suggestion is sustained by the fact that the immuno-gold labelling of the ER for lysosomal α -glucosidase is quantitatively normal,

whereas the Golgi complex and the more distal compartments of the lysosomal transport route are clearly deficient in lysosomal α -glucosidase. This explains why transfection of COS cells with $\Delta G2$ does not lead to a significant increase of the lysosomal α -glucosidase activity.

Mutation analyses of the glycosylation sites of few other lysosomal proteins have been reported. For example site directed mutagenesis was used to determine the essential glycosylation sites of human β -hexosaminidase B (Sonderfeld-Fresco and Proia 1989). In this enzyme four of the five potential sites were found to be glycosylated. Elimination of each site individually had no effect on lysosomal targeting or catalytic function. In addition to these studies two naturally occurring glycosylation site mutations in lysosomal proteins have been reported. One concerns arylsulfatase A. Normally, two of the three potential sites are glycosylated. But individuals carrying the pseudodeficiency allele have lost one of the two utilized sites by a mutation changing Asn 350 to Ser (Gieselmann et al. 1989). However, the introduction of this mutation into wild type cDNA did not affect the catalytic function nor the stability of the encoded enzyme. The reduced arylsulfatase A activity leading to this pseudodeficiency is caused by a mutation abolishing the first polyadenylation signal. As a consequence the mRNA species is labile which explains the severely diminished rate of enzyme synthesis (Gieselmann et al. 1989). The second mutation of a glycosylation site was found in saposin B. The mutation changes Thr 23 to Ile which eliminates the only possible site. The mutant protein is believed to be rapidly degraded (Rafi et al. 1990; Kretz et al. 1990). These examples and our own experiments demonstrate the differential function of glycosylation sites.

The incorporation of [32 P]P_i in the different biosynthetic forms of lysosomal α -glucosidase allows us to make an estimate on the minimal number of carbohydrate chains that are phosphorylated. Although each mutant is missing a different glycosylation site it appears that all mutant precursor proteins are phosphorylated. From this we conclude that at least two of the seven carbohydrate chains contain the mannose 6-phosphate recognition marker. Furthermore, it is evident that at least one of the five carbohydrate chains located within the boundaries of the 76 kD polypeptide is phosphorylated since also the mature enzyme is phosphate labelled. The maturation defect of mutant $\Delta G2$ prevents the assessment of phosphorylation at site Asn 233 and thereby hampers a more detailed analysis of the phosphorylation sites by site directed mutagenesis. A further identification of the phosphorylation sites via sequence analysis of phosphorylated peptides is in progress.

Acknowledgements

The authors like to thank Alvin Chan for technical assistance, Lies Hoefsloot and Marianne Hoogeveen-Westerveld for stimulating discussion and Tom de Vries Lentsch for the preparation of photographic illustrations.

Materials and methods

Construction of mutants

The Muta-GeneTM *in vitro* Mutagenesis kit from Bio-Rad (Richmond, USA) was used to carry out site directed mutagenesis, as described by Kunkel (1985). The oligonucleotides used to alter the seven potential glycosylation sites are listed in Table II. They were synthesized on an Applied Biosystems 381A DNA synthesizer. Site directed mutagenesis and cloning of the mutant cDNA's in the eukaryotic expression vector pSG5 (Green et al. 1988) was performed exactly as described previously (Hermans et al. 1991b).

Table II

Oligonucleotides used for mutagenesis.

mutant	oligonucleotide*
ΔG1	5' AAGCTGGAG <u>C</u> AGCTGAGCTCC 3'
ΔG2	5' GTGCTGCTG <u>C</u> AGACGACGGTG 3'
ΔG3	5' GTGGTGGAG <u>C</u> AGATGACCAGG 3'
ΔG4	5' TTCATCACCC <u>A</u> GGAGACCGGC 3'
ΔG5	5' TTCCTGGGC <u>C</u> AGACCTCAGAG 3'
ΔG6	5' CTGGCCAGG <u>C</u> AGAACACGATC 3'
ΔG7	5' CCTGTCTCC <u>C</u> AGTTCACCTAC 3'

* The altered nucleotides are underlined

Transient expression in COS cells

COS-1 cells (Gluzman, 1981) were cultured in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics, at 37 °C. The transfection protocol was as described before (Hoefsloot et al. 1990). The culture medium was collected and the cells were harvested at 90 h after transfection. Cell homogenates were made by repeated freezing and thawing of cell pellets in distilled water.

The activity of lysosomal α -glucosidase in the cell homogenate and the culture medium was measured with 4-methylumbelliferyl- α -D-glucopyranoside (4-MU) as described before (Reuser et al. 1978). The protein concentrations of cell homogenates were determined with the use of the BCA protein assay kit (Pierce). Lysosomal α -glucosidase was immunoprecipitated from culture media using a rabbit polyclonal antiserum against human lysosomal α -glucosidase in combination with *Staphylococcus aureus* membranes (Bethesda Research Laboratories) and analyzed by immunoblotting (Reuser et al. 1987). To characterize the intracellular forms of lysosomal α -glucosidase COS cells were labelled for 2 h with [³H]-leucine (190 μ Ci/mmol)(Amersham U.K.) at 65 h after transfection and lysosomal α -glucosidase was immunoprecipitated either directly (pulse) or after 16 h of chase. The different molecular species of lysosomal α -glucosidase were separated by SDS-PAGE (8% acrylamide, 1% crosslink unless indicated otherwise) as described (Reuser et al. 1985).

Phosphorylation of lysosomal α -glucosidase

COS cells were transfected as described above. After 90 h the cells were pre-incubated for 1 h in phosphate-free DMEM to which FCS, dialysed against 0.9% NaCl, was added in a final concentration of 4%. The medium was then refreshed and carrier-free [32 P]P_i phosphate (Amersham) was added at a concentration of 80 μ Ci/ml. The cells were pulse-labelled for 6 h and harvested either directly or after a subsequent chase of 12 h. Lysosomal α -glucosidase was immunoprecipitated from cell extracts as described (Reuser et al. 1985) and analyzed by SDS-PAGE.

In vitro transcription and translation

Wild type and mutant cDNA's cloned in the expression vector pSG5 were linearized with BglII and used as a template in the transcription reaction. The T7 promoter was used for transcription. The reaction was allowed to proceed for 1.5 h at 40 °C in 40 mM Tris/HCl, pH 7.5, containing 6 mM MgCl₂, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 1 unit RNase inhibitor (Promega), 0.8 mg/ml bovine serum albumin, 500 μ M ATP, CTP and UTP, 50 μ M GTP, 500 μ M dGpppG, and 1 μ g of DNA template with 20 units T7 polymerase (Boehringer Mannheim). The template was removed by adding 1 unit RNase free DNase (Boehringer Mannheim). The excess of nucleotides was removed by Sephadex G50 filtration.

A 0.2 μ g aliquot of RNA was used for *in vitro* translation in a rabbit reticulocyte lysate system (Promega) containing 54 μ Ci of [35 S]-methionine (1130 Ci/mmol, Amersham International) in the presence of canine pancreatic microsomal membranes, according to the instructions of the manufacturer. Radioactively labelled lysosomal α -glucosidase was immunoprecipitated and separated by SDS-PAGE as described above.

Immunocytochemistry

Immunocytochemistry on transiently transfected COS cells was performed exactly as described before (Hoefsloot et al. 1990).

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Chapter 7

Lysosomal acid α -glucosidase expressed in polarized kidney epithelial cells is targeted to lysosomes and secreted preferentially at the apical cell surface

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Summary

The lysosomal enzyme acid α -glucosidase is evolutionary related to the intestinal brush border enzymes sucrase-isomaltase. Possibly due to the structural homology, acid α -glucosidase is not restricted in its localization to the lysosomes, but is also present on the microvilli of intestinal epithelium and polarized CaCo-2 cells. A precursor form of acid α -glucosidase is secreted preferentially from the apical surface of the latter cells. We have transfected MDCK-II (Madin Darby canine kidney cells resembling epithelium of the distal tubuli) and LLC-PK₁ cells (representing proximal tubule cells) with full length acid α -glucosidase cDNA to study in detail the expression and routing of the enzyme in polarized cells. Both transiently and stably transfected cells were used.

The proteolytic and carbohydrate modifications of the acid α -glucosidase precursor were found to be similar as in non-polarized human fibroblasts. The early 110 kDa precursor was membrane bound, whereas the processed forms of 95 kDa and 76 kDa were soluble. The majority of enzyme was targeted to the lysosomes, but a fraction of newly synthesized acid α -glucosidase was routed preferentially to the apical cell surface, and secreted. Hardly any acid α -glucosidase appeared to be associated with the apical plasma membrane. The data suggest that the precursor of acid α -glucosidase is released from its membrane anchor before it reaches the cell surface. Therefore, it seems that the apical sorting signal is not located in the transmembrane domain of the early enzyme precursor. We propose instead that the physical properties of acid α -glucosidase make the enzyme enter the apical secretory route.

Introduction

The epithelial cells lining the proximal and distal tubules of the kidney are polarized. The apical plasma membrane domain facing the lumen of the tubule is separated from the basolateral domain by tight junctions, and the lipid and protein composition of the two cell surface domains is characteristically different (Rodriguez-Boulan, 1983; Simons and Fuller, 1985; Van Meer and Simons, 1986; Brown, 1989; Handler, 1989). An efficient sorting mechanism ensures delivery of plasma membrane components to their appropriate apical or basolateral destinations. The molecular signals involved in this protein sorting have been studied most extensively in MDCK-II cells (Madin-Darby canine kidney), with typical characteristics of a distal tubule cell (Cereijido et al. 1978). It has been established that the sorting takes place in the trans Golgi network from where distinct transport vesicles depart to either side of the cell (Wandinger-Ness et al., 1990). Basolateral transport of plasma membrane proteins was originally thought to occur by default, but evidence is accumulating that it is guided by signals in the cytoplasmic tails of the basolateral proteins (Casanova et al., 1991; Mostov et al., 1992). Similar built-in signals directing apical transport have not yet been identified, but there are some indications that these are localized in the luminal ectodomain of the apical plasma membrane proteins (Vogel et al., 1992). Another cell line used to study polarized transport is LLC-PK₁, which is derived from pig kidney and has morphological features of proximal tubule cells (Rabito and Karish, 1982).

In the studies referred to above much attention was given to the differential transport of influenza virus hemagglutinin and vesicular stomatitis virus G protein. The polymeric immunoglobulin receptor was studied for its involvement in the transcytosis of IgA and IgM across various epithelia (Casanova et al., 1991; Okamoto et al., 1992). Also enzymes of the apical plasma membrane of intestinal epithelial cells such as aminopeptidase N and dipeptidyl peptidase IV (DPP-IV) were used frequently as a model (Casanova et al., 1991; Low et al., 1991). Among the variety of other proteins studied were some resident glycoproteins of the lysosomal membrane (Nabi et al., 1991; Harter and Mellman, 1992). No studies have been reported yet on the transport of lysosomal enzymes in polarized kidney epithelial cells.

This study reports on the biosynthesis and transport of lysosomal acid α -glucosidase in transfected MDCK-II and LLC-PK₁ cells. The enzyme was chosen for its peculiar features. Acid α -glucosidase is synthesized as a membrane bound precursor which is solubilized proteolytically during maturation (Tsuiji and Suzuki, 1987; Hoefsloot et al., 1990; Wisselaar et al., 1993). At least two of the seven N-linked carbohydrate side chains of the acid α -glucosidase precursor are phosphorylated at mannose residues (Hasilik and Neufeld, 1980b; Hermans et al., 1992). The phosphorylated precursor can

bind to the mannose 6-phosphate receptor via which lysosomal enzymes are sorted and transported to the lysosomes (Reuser et al., 1984). But, acid α -glucosidase is not strictly dependent on the mannose 6-phosphate receptor for its lysosomal transport since the activity of acid α -glucosidase is only partially reduced when the mannose 6-phosphate recognition system is not operative as is the case in I-cell disease (Van Dongen et al., 1985; Tsuji et al., 1988). An alternative transport system relying on membrane association has been suggested for acid α -glucosidase. Another peculiar feature of acid α -glucosidase is the reported extralysosomal localization at the apical plasma membrane domain of human intestinal and renal epithelium (Fransen et al., 1988; Oude Elferink et al., 1989; Willemsen et al., 1991), at the apical side of polarized CaCo-2 cells (Klumperman et al., 1991), and at the cell surface of non-polarized transiently transfected COS-1 cells (Hoefsloot et al., 1990). In addition it was reported that lysosomal acid α -glucosidase, is preferentially secreted from the apical surface of the intestinal CaCo-2 cells (Klumperman et al., 1991).

Our results show that acid α -glucosidase is transported to the lysosomes of transfected MDCK-II and LLC-PK₁ cells and that a fraction of precursor molecules is secreted from the apical more than from the basolateral cell surface. A similar preference for apical secretion was demonstrated for the endogenous lysosomal enzyme β -hexosaminidase. No evidence was obtained for apical membrane association of acid α -glucosidase.

Results

Expression of acid α -glucosidase in transiently transfected renal cells.

Subconfluent MDCK-II and LLC-PK₁ monolayers were transfected with an eukaryotic expression vector containing full length acid α -glucosidase cDNA (pSHAG 2). Indirect immunofluorescence was performed 72 hours later when the monolayers had reached confluency, and the cells were polarized. In cells expressing acid α -glucosidase (about 2% of the total population) a typical punctate lysosomal labelling pattern was observed (Fig. 1). The lysosomal localization of acid α -glucosidase was confirmed with immuno-electron microscopy. Not only the lysosomes but also the transport route to the lysosomes including the rough endoplasmic reticulum and the Golgi complex were labelled (Figs 2 A,B). No enzyme was detectable at the apical nor at the basolateral plasmamembrane. Background labelling was negligible as shown in sections which were incubated with pre-immune serum (Fig. 2C).

The relative concentration of the different molecular forms of acid α -glucosidase in MDCK-II cells at 48 hours after transfection is shown on the immunoblot of Fig. 3.

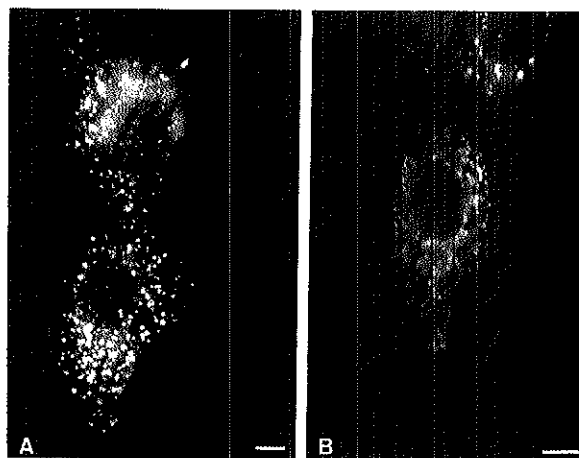


Figure 1. Immunocytochemical localization of acid α -glucosidase in transfected MDCK-II (A) and LLC-PK₁ (B) cells. Cells were fixed and stained with a polyclonal antiserum against acid α -glucosidase as described in Materials and Methods. Bar represents 10 μ m.

Major species of 95 kDa and 76 kDa were present intracellularly in approximately equal amounts. The 95 kDa species is a known long lived processing intermediate, and the 76 kDa species a long lived end stage form of lysosomal acid α -glucosidase (Hasilik and Neufeld, 1980; Reuser et al., 1985). The precursor of acid α -glucosidase with a mass of approximately 110 kDa was hardly detectable intracellularly but immunoprecipitable from the culture medium. Virtually the same results were obtained with LLC-PK₁ cells expressing acid α -glucosidase transiently (data not shown).

Synthesis of acid α -glucosidase in stably transfected MDCK-II and LLC-PK₁ cells.

The transfection frequency was low (2%), which made it difficult to study the biosynthesis of acid α -glucosidase in the mixed population of transfected and untransfected cells. Therefore, stably transfected cell lines were produced via clonal selection. The acid α -glucosidase activities of a number of different clones obtained by transfection of MDCK-II and LLC-PK₁ cells are listed in Table I. All clones had the same morphology and the same extent of polarization as the untransfected parental cell lines (Fig. 4).

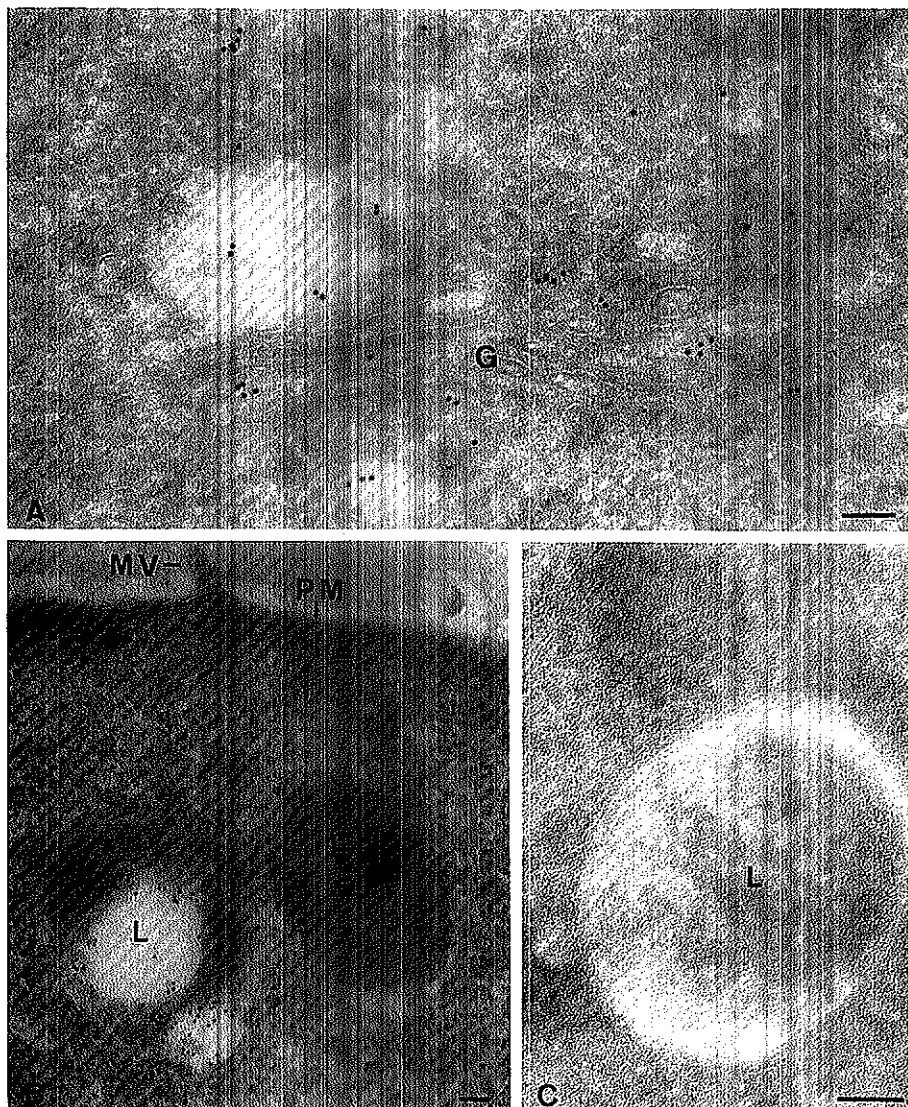


Figure 2. Subcellular localization of acid α -glucosidase in transfected MDCK-II cells. Cryosections were prepared at 72 hours after transfection and incubated with a polyclonal antiserum (A and B) or with pre-immune serum (C). G, Golgi complex; L, Lysosome; PM, Plasma Membrane; MV, Microvillus. Bar represents 0.1 μ m.

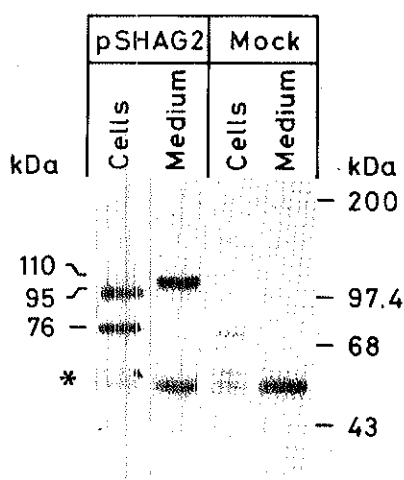


Figure 3. Molecular characterization of the acid α -glucosidase species present in transfected MDCK-II cells via Western blotting. Acid α -glucosidase was immunopurified from transfected MDCK-II cells and from the culture medium and applied to SDS-PAGE. The proteins were blotted to nitrocellulose and visualized with antibodies against acid α -glucosidase in combination with 125 I-protein A (pSHAG 2). As a negative control, MDCK-II cells were transfected with a cDNA construct of *E. coli* β -galactosidase (Mock). (* represents immunoglobulins).

Table I

Acid α -glucosidase activity of stably transfected cell lines.

Cell line	Acid α -glucosidase activity*
MDCK II (untransfected host)	8.3
MDCK-GAA 285	50.9
MDCK-GAA 2816	52.1
LLC-PK ₁ (untransfected host)	20.3
LLCPK-GAA 82	1764.0
LLCPK-GAA 819	1975.0
LLCPK-GAA 98	656.0
LLCPK-GAA 915	677.0
LLCPK-GAA 1711	877.0

*Expressed as nmol 4-MU/mg. protein/hour.



Figure 4. Electron micrographs of monolayers of acid α -glucosidase expressing MDCK-GAA 285 (A) and LLC-PK-GAA 819 (B) cells cultured on filters. Cells were fixed and embedded as described in Materials and Methods. The cells are highly polarized. (A) Bar represents 1 μ m, (B) Bar represents 0.5 μ m.

The proteolytic processing and glycosylation of acid α -glucosidase was studied in clone MDCK-GAA 285 grown on filters (Fig. 5). The 110 kDa precursor was the predominant molecular form of acid α -glucosidase after a two hours labelling period (0 hr chase). This precursor was processed within three hours to a transient 100 kDa species and twenty four hours later to long lived species of 95 kDa and 76 kDa. At this time point, labelled 110 kDa precursor did no longer exist. Figs 6 and 7 show on immunoblots the steady state concentration of the different intra- and extracellular forms of acid α -glucosidase in stably transfected MDCK-II and LLC-PK₁ cell lines after five days of confluency. The 95 kDa species was the major form of acid α -glucosidase in clone MDCK-GAA 285 whereas the LLC-PK-GAA clones expressed relatively more 76 kDa

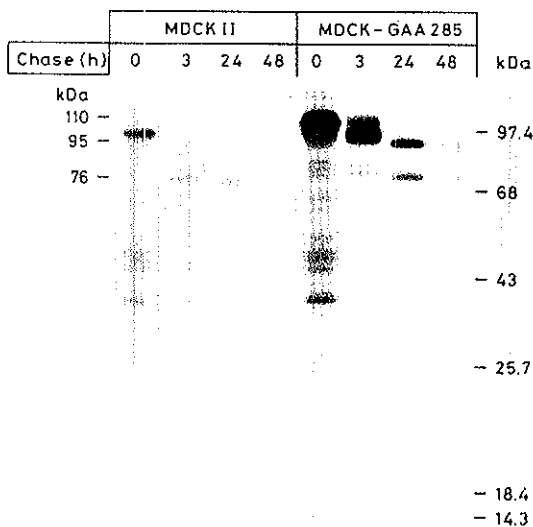


Figure 5. Biosynthesis of acid α -glucosidase in MDCK-GAA 285 cells. Acid α -glucosidase was labelled for 2 hours by incorporation of ^3H -leucine and immunoprecipitated from the cell homogenate, either directly (0 hour of chase) or after a 3, a 24 or a 48 hours chase period. Un-transfected MDCK-II cells were labelled to visualize endogenous acid α -glucosidase production.

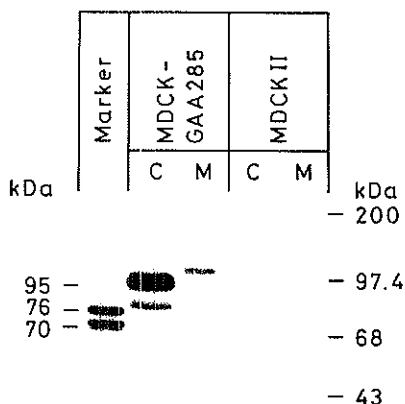


Figure 6. Demonstration of the bio-synthetic forms of acid α -glucosidase in MDCK-GAA 285 cells. Acid α -glucosidase was immunopurified from MDCK-GAA 285 cells and from untransfected MDCK-II cells and from the respective culture media and applied to SDS-PAGE. The 95 kDa, 76 kDa and 70 kDa species of acid α -glucosidase purified from human placenta were applied directly to the gel as marker. The immunoblot analysis was performed as described in Fig. 3. C, cells; M, medium.

Figure 7. Molecular characterization of acid α -glucosidase expressed in stably transfected LLC-PK₁ cells and their culture media. The experiment was performed as described in the legend of Fig. 3. Un-transfected LLC-PK₁ cells (middle lanes) were used as a control. C, cells; M, medium.

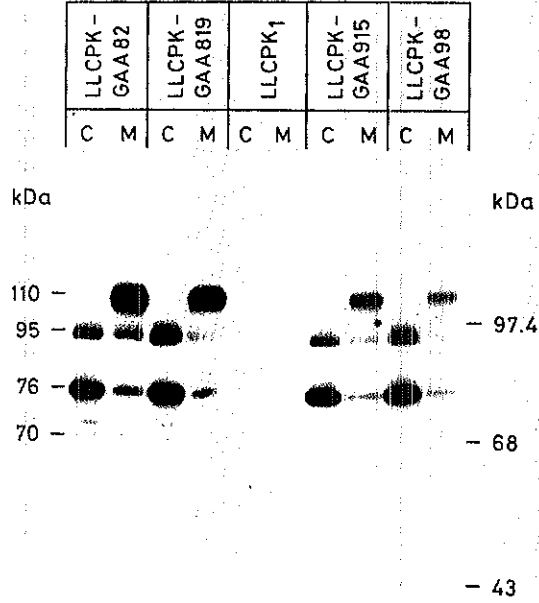
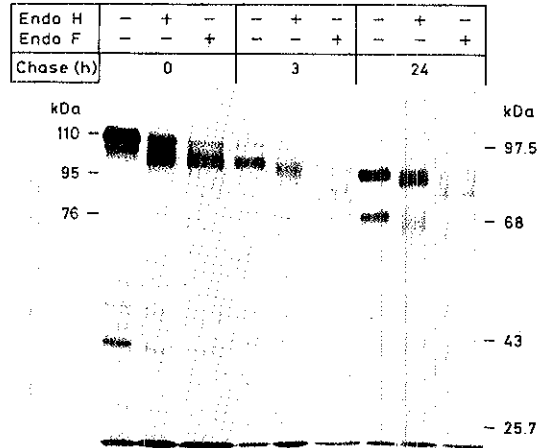


Figure 8. The glycosylation of acid α -glucosidase in MDCK-GAA 285 cells. Cells were labelled for 2 hours with ³H-leucine whereafter acid α -glucosidase was analysed as described in the legend of Fig. 5. Immunoprecipitated enzyme was treated with Endo H or Endo F as indicated.



than 95 kDa enzyme. Both these molecular species were also found in the medium of LLC_{PK}-GAA clones, but this was due to leakage following cell death rather than to secretion. The 110 kDa precursor was secreted by all stably transfected clones.

The oligosaccharide modification during posttranslational processing was studied by Endo H and Endo F digestion. Endo H cleaves oligosaccharides of the high-mannose type only, whereas Endo F cleaves both high-mannose as well as complex type of N-linked sugar structures. The 110 kDa precursor (0 hr chase) had the same sensitivity for Endo H and Endo F and the carbohydrate chains were therefore exclusively of the high-mannose type (figure 8). The 100 kDa (3 hr chase) and 95 kDa (24 hr chase) species that were formed later had acquired complex type of oligosaccharides and were more sensitive to Endo F than to Endo H (Fig. 8).

Previous studies had shown that the 110 kDa precursor of acid α -glucosidase was largely membrane bound in human fibroblasts (Tsuiji and Suzuki, 1987) and human placental tissue (Tsuiji and Suzuki, 1988) but also in transiently transfected COS-1 cells (Hoefsloot et al., 1990). In order to analyse the existence of membrane bound forms of acid α -glucosidase in the polarized kidney epithelial cell lines we performed a Triton X-114 phase separation using clone MDCK-GAA 285 (Fig. 9). The 110 kDa precursor of acid α -glucosidase was exclusively present in the detergent phase, whereas intermediate and mature forms segregated in the water phase, indicating that also in these cells the precursor was membrane bound.

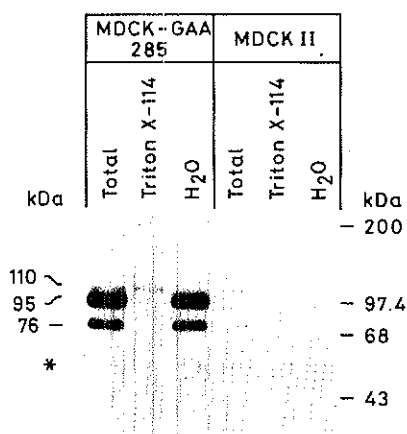


Figure 9. Triton X-114 phase separation of the different molecular forms of acid α -glucosidase synthesized in MDCK-GAA 285 cells. A Triton X-114 phase separation was carried out according to Bordier (1981). Acid α -glucosidase was immunopurified from the detergent (Triton X-114) and water phase as well as from the total cell homogenate. The enzymes were visualized on immunoblots after SDS-PAGE as described in Fig. 3.

Intracellular localization of acid α -glucosidase.

The intracellular localization of acid α -glucosidase, in clones MDCK-GAA 285 and LLCKP-GAA 819 is shown in Figs 10 and 11, respectively. Lysosomes were strongly labelled, whereas the rough endoplasmic reticulum and the Golgi complex were weakly labelled resembling the distribution of the enzyme in human fibroblasts (Reuser et al., 1987). Some label was occasionally encountered on the apical microvilli of the transfected proximal (LLCPK-GAA 819) and distal (MDCK-GAA 285) tubule cells, but the significance was questionable. Labelling of the basolateral membrane was equally weak, but acid α -glucosidase was clearly contained in vesicles just below the apical cell surface.

Polarized secretion of acid α -glucosidase by stably transfected renal cell lines.

To study the polarity of acid α -glucosidase secretion, cells were grown to confluence on filters, thus allowing separate analysis of enzyme activity in the apical and basolateral media. The secretion of endogenous acid α -glucosidase and β -hexosaminidase was studied as a control (Figs 12 and 13). Endogenous acid α -glucosidase and β -hexosaminidase were secreted in both directions (apical and basolateral) by the untransfected MDCK-II and LLC-PK₁ cells. In stably transfected MDCK-II and LLC-PK₁ cell lines there was a strong prevalence of the apical secretion pathway.

It is noteworthy that overexpression of acid α -glucosidase had no effect on the secretion of endogenous β -hexosaminidase (Figs 12 and 13).

The mannose-6-phosphate receptor function in untransfected MDCK-II and LLC-PK₁ cells.

To study the possible involvement of the mannose 6-phosphate receptor in transport of acid α -glucosidase to the apical or basolateral cell surface we have localized the receptor indirectly by studying its capacity to bind and endocytose exogenously administered phosphorylated acid α -glucosidase purified from bovine testis (Reuser et al., 1984). Administration of enzyme at the basolateral side of polarized MDCK-II and LLC-PK₁ cells on filter resulted in very efficient uptake (Table II). Uptake was mediated by the mannose 6-phosphate receptor, because it was blocked by 7.5 mM mannose 6-phosphate. When the enzyme was added to the apical side of the cells very little was internalized. Uptake could not be inhibited by mannose 6-phosphate indicating that the enzyme entered the cells at the apical side via fluid phase endocytosis.

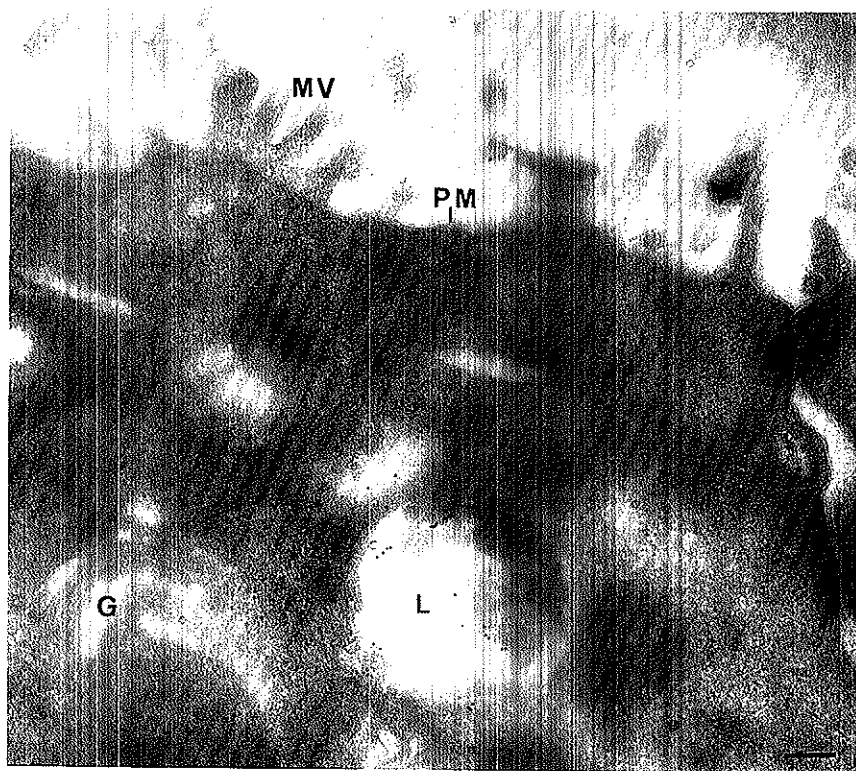


Figure 10

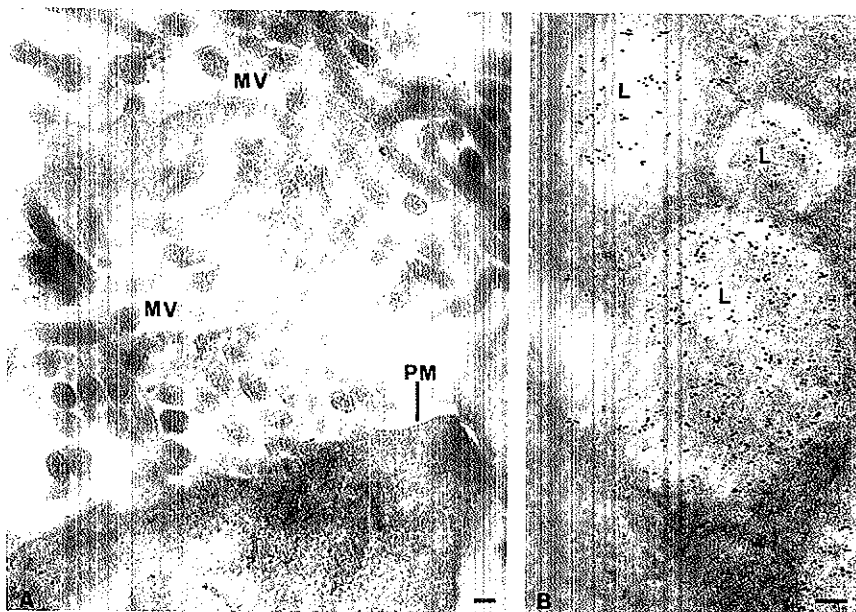


Figure 11

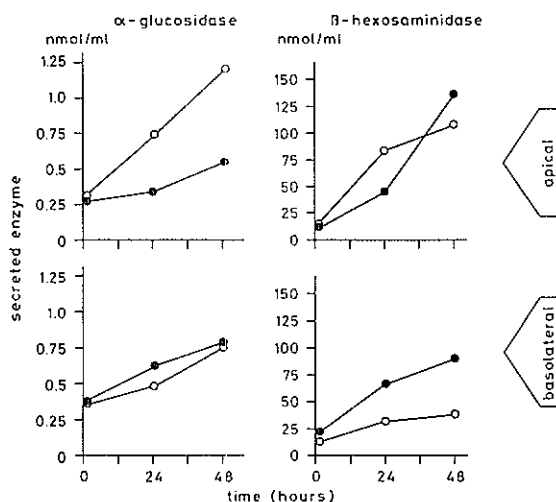


Figure 12. Secretion of acid α -glucosidase by filter grown stably transfected MDCK-GAA 285 cells (○) in comparison to filter grown MDCK-II cells (●). As a control the secretion of β -hexosaminidase was measured.

Fig. 10 and 11 on opposite page.

Figure 10.

Immunoelectron microscopical localization of acid α -glucosidase in filter grown MDCK-GAA 285 cells. Cryosections were prepared and incubated with a polyclonal antiserum. G, Golgi complex; L, Lysosome; PM, Plasma Membrane; MV, Microvilli. A junctional area is present on the right. Bar represents 0.2 μ m.

Figure 11.

Subcellular localization of acid α -glucosidase in filter grown LLC-PK-GAA 819 cells. The procedure of staining was similar as described in the legend of Fig. 10. L, Lysosomes; PM, Plasma Membrane; MV, Microvilli. Bar represents 0.1 μ m.

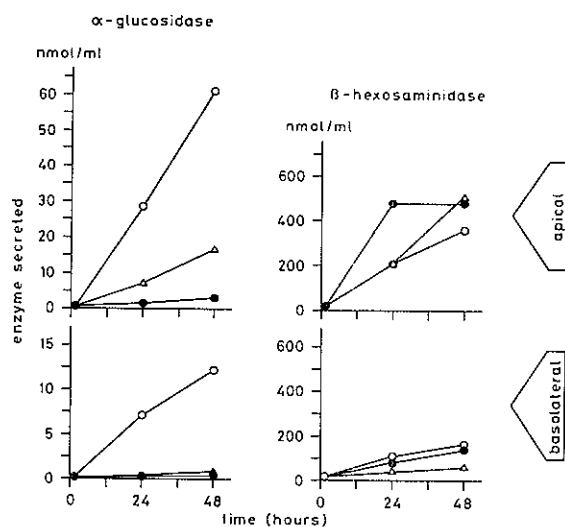


Figure 13. Polarized secretion of acid α -glucosidase in stably transfected LLC-PK₁ cells. The same procedure was used as in the legend of Fig. 12. (●) LLC-PK₁, (○) LLC-PK-GAA 819, (△) LLC-PK-GAA 1711.

Table II

Uptake of bovine testis acid α -glucosidase by MDCK-II and LLC-PK₁ cells.

Side of enzyme administration	M6P addition	α -Glucosidase		β -Hexosaminidase	
		MDCK-II	LLC-PK ₁	MDCK-II	LLC-PK ₁
—		33.6	77.9	3128	6700
Apical	—	42.8	93.3	3089	6711
Apical	+	30.3	91.0	3018	6947
Basolateral	—	87.0	191.9	2949	6088
Basolateral	+	34.9	82.1	3037	6801

*Expressed as nmol 4-MU/mg protein/hour. M6P = mannose 6-phosphate.

Discussion

Suggestive evidence has been presented for the apical targeting of the lysosomal enzyme acid α -glucosidase in polarized epithelial cells. The acid α -glucosidase precursor was visualized immuno-cytochemically in the brush border of intestinal epithelium (Fransen et al., 1988). The results were confirmed by semi-quantitative analysis, and the specificity of apical sorting of acid α -glucosidase was demonstrated by the near absence of two other lysosomal enzymes in the brush border (Willemssen et al., 1991). A similar apical localization of acid α -glucosidase was reported in the adenocarcinoma, CaCo-2, cell line. Studying these cells it was observed, in addition, that the acid α -glucosidase precursor was secreted preferentially from the apical cell surface (ratio apical versus basolateral is 7:3) (Klumperman et al., 1991). In contrast, three other lysosomal enzymes, β -hexosaminidase, β -glucuronidase and cathepsin D were secreted with preference from the basolateral cell surface (ratio 4:6) (Klumperman et al., 1991). In analogy to the intestinal brush border localization, acid α -glucosidase was also observed on the microvilli of the epithelial cells lining the proximal tubules of the kidney. This polar localization was brought into relation with the excretion of lysosomal enzymes in the urine (Oude Elferink et al., 1989). Indeed, active secretion is expected since about 50% of the total acid α -glucosidase in human urine represents a phosphorylated high molecular weight enzyme precursor, the amount of which is too high to be derived from dead kidney cells (Oude Elferink et al., 1989). Taken together, these findings suggest that certain polarized cell types have a mechanism to sort acid α -glucosidase preferentially to the apical membrane domain. According to the current ideas about protein sorting, the assumption can be made that apical transport of acid α -glucosidase is triggered by molecular signals encoded in the protein structure or alternatively that apical transport of acid α -glucosidase occurs by default. To address this question we have studied the biosynthesis and transport of acid α -glucosidase in transfected polarized MDCK-II and LLC-PK₁ cell lines that are well characterized and are widely used as model system. The endogenous acid α -glucosidase production is low in these cell lines and the antiserum used to detect the human cDNA encoded enzyme has a very low titer for the endogenous acid α -glucosidase.

The proteolytic processing of the acid α -glucosidase precursor in polarized MDCK-II and LLC-PK₁ cells was found to be similar as in transfected non-polarized COS-1 cells and human fibroblasts. A difference, however, was the existence of a relatively stable 100 kDa intermediate. This intermediate was short lived in the latter two cell types and only revealed when proteolytic processing is retarded by the thiol proteinase inhibitors leupeptin and E-64 (Wisselaar et al., 1993). Since it is the same acid α -glucosidase that is expressed in all three cell types we conclude that either the

concentration of modifying proteinases is different in MDCK-II and LLC-PK₁ cells compared to COS-1 cells and fibroblasts or the rate of acid α -glucosidase transport.

The acid α -glucosidase precursor produced in stably transfected MDCK-II cells is membrane bound as it is in fibroblasts (Tsuji and Suzuki, 1987) and transiently transfected COS-1 cells (Hoefsloot et al., 1990). We have recently shown that the membrane anchorage in the COS-1 cells is effected by an uncleaved signal peptide (Wisselaar et al., 1993). It was proposed that the localization of acid α -glucosidase along the microvilli of intestinal epithelial and CaCo-2 cells was caused by inefficient release of the precursor from its membrane anchor. This suggestion was done in analogy to the typical membrane association of the structurally homologous intestinal brush border enzyme complex of sucrase and isomaltase, which is a type I glycoprotein with an uncleaved signal peptide (Hunziker et al., 1986). In contrast to our expectation there appeared to be hardly any acid α -glucosidase associated with the apical plasma membrane of the transfected MDCK-II and LLC-PK₁ cells. From this we infer that all precursor molecules have lost their signal peptide by the time they arrive at the cell surface, and are secreted as a result. Also this difference in the plasma membrane association of acid α -glucosidase in MDCK-II and LLC-PK₁ cells compared to COS-1 cells can be explained by a different protease activity or rate of transport in the various cell types.

It is noteworthy that acid α -glucosidase is secreted by both the LLC-PK₁ as well as the MDCK-II clones whereas in vivo it are mainly the proximal tubule cells that secrete acid α -glucosidase. A second difference between the in vitro and in vivo situation is the unexpected lack of acid α -glucosidase on the microvilli of transfected LLC-PK₁ cells whereas the proximal tubule cells have a strong brush border labelling in vivo (Oude Elferink et al., 1989). The secretion of acid α -glucosidase by transfected MDCK-II and LLC-PK₁ cells is in itself not surprising. Other cell types like fibroblasts, myoblasts and epithelial cells are known to secrete a small percentage of the newly synthesized lysosomal enzyme pool. It is of interest, however, that the secretion of acid α -glucosidase by MDCK-II and LLC-PK₁ is polarized with preference for the apical cell surface. The ratio of apical versus basolateral secretion was independent of the acid α -glucosidase expression level. From 82 to 98% of acid α -glucosidase activity was secreted from the apical cell surface of LLC-PK₁ clones. For transfected MDCK-II cells this figure was approximately 80%. Also endogenous β -hexosaminidase was preferentially secreted from the apical cell surface (70-90%) and the direction of secretion was not significantly influenced by the level of acid α -glucosidase expression. From this we conclude that neither the basolateral nor the apical transport pathways are saturated by the high level expression of acid α -glucosidase.

For long it was thought that molecular sorting signals were mandatory for apical transport of plasma membrane proteins in MDCK-II cells and that basolateral transport

did occur by default. However, the only apical sorting signal identified thus far is the carboxy-terminal glycosyl-phosphatidylinositol (GPI) anchor of certain plasma membrane glycoproteins. As a sorting mechanism it has been suggested that GPI-anchored proteins cluster with glycopospholipids in vesicles leaving the trans Golgi network with an apical destination (Mostov et al., 1992). How other apical membrane proteins are sorted is still unknown (Bartles and Hubbard, 1988; Simons and Wandinger-Ness, 1990; Mostov et al., 1992). Meanwhile, more evidence is accumulating for the existence of a basolateral sorting mechanism. Sorting appears to be directed by a conformational signal in the cytoplasmic domain of transmembrane proteins. That signal is in some proteins similar as, but in others distinct from the tyrosine dependent signal for endocytosis (Brewer and Roth, 1991; Hopkins, 1991; Le Bivic et al., 1991; Hunziker et al., 1991; Mostov et al., 1992). For the transferrin receptor, for lysosomal acid phosphatase and for the LDL receptor it was recently shown that the tyrosine essential for endocytosis occurs in the structural context of a tight β -turn (Collawn et al., 1990; Bansal and Gierasch, 1991; Eberle et al., 1991).

Much less information is available on the molecular signals involved in the apical versus basolateral sorting of secretory proteins. Some endogenous secretory proteins of MDCK-II cells are preferentially sorted to the basolateral surface, while others are released from the apical surface (Kondor-Koch et al., 1985; Gottlieb et al., 1986; Bartles and Hubbard, 1988). This would suggest that sorting signals exist, indeed, also for this category of proteins. But, on the other hand, the apical versus basolateral targeting of secretory proteins is rarely absolute and some exogenous proteins are secreted from both surfaces in about equal amounts. Our own experiments on the posttranslational modification and routing of acid α -glucosidase in transfected MDCK-II and LLC-PK₁ cells indicate at first sight that the enzyme has an apical sorting signal. This signal has to be distinct from the mannose 6-phosphate recognition marker since the mannose 6-phosphate receptor in MDCK-II cells is targeted to the basolateral instead of the apical cell surface. This was earlier demonstrated by Prydz et al. (1990) and confirmed in our experiments by the uptake of phosphorylated bovine testis acid α -glucosidase from the basolateral but not from the apical medium.

Following the current concepts one can assume that an apical targeting signal is imprinted in the conformation of acid α -glucosidase. On the other hand the fundamental question remains of why enzymes with a lysosomal destination and function would require such a signal. Therefore, we like to consider the possibility that the preferential apical secretion of lysosomal acid α -glucosidase and β -hexosaminidase in MDCK-II and LLC-PK₁ cells occurs actually by default. The mechanism could be the following. Secretory and plasma membrane proteins arrive together in the trans Golgi network. The GPI anchored proteins are thought to associate with clustered glycopospholipids in vesicles

directed to the apical cell surface. The transmembrane glycoproteins are sorted, whereby those with a basolateral targeting signal in the cytoplasmic tail are sequestered in vesicles with a basolateral destination. Those without signal are collected in vesicles with an apical destination. The intestinal brush border enzymes sucrase-isomaltase, dipeptidylpeptidase-IV and aminopeptidase-N which are anchored via their uncleaved signal peptide could belong to the latter category, since they are preferentially transported to the apical cell surface when expressed in MDCK-II cells. Secretory proteins without specific targeting signal may segregate in either type of vesicle depending on their specific physical properties, and the cell type specific capacity of the apical and basolateral transport systems.

This model fits with the situation often encountered i.e. that the direction of transport is rarely exclusively apical or basolateral, and that the direction can vary per cell type (Bartles and Hubbard, 1988; Wessels et al., 1990). According to this model the apical secretion of the anchor minus mutant of aminopeptidase N in MDCK-II cells can occur by default rather than being directed by a well defined apical sorting signal in the ectodomain of the truncated protein (Vogel et al., 1992). Expression of mutant forms of acid α -glucosidase or mutated exogenous secretory proteins in MDCK-II and LLC-PK₁ cells may help to test the validity of this model. Mutations affecting the conformation or the physical properties of the enzyme are hereby of particular interest.

Acknowledgements

The technical advise of Rob Willemsen concerning the performance of immuno-electronmicroscopy was much appreciated and the stimulating discussions with Marian Kroos and Ben Oostra. Pim Visser, Tom de Vries Lentsch and Ruud Koppenol were very helpful with preparing the figures and the photographic artwork, and Jeannette Lokker with secretarial assistance. Financial support was obtained from the Netherlands Organization for Scientific Research, subsidiary of Medical Sciences.

Materials and methods

Cell Culture.

MDCK cells (strain II; canine kidney distal tubulus), from the 6th passage on were maintained at 37°C under 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories) supplemented with 5% fetal calf serum, 10 mM Hepes and antibiotics (120 μ g of streptomycin and 100 IU penicillin/ml; Gibco). To induce polarization 7x10⁵ cells were seeded on polycarbonate membranes (24.5 mm diameter, 3 μ m pore size, Transwell chambers; Costar) and cultured for 4 days with changes of medium after 2 days.

LLC-PK₁ cells (pig kidney proximal tubulus), from passage 205th on were maintained in DMEM supplemented with 10% fetal calf serum and antibiotics. Polarization of these cells was induced by seeding 7x10⁵ cells on collagen coated Costar filters (24.5 mm diameter, 0.4 μ m pore size).

Transfection Procedures.

For transient expression of acid α -glucosidase in MDCK II or LLC-PK₁ cells a modification of the calcium-phosphate method was used (Graham and Van der Eb, 1973). In short, 40 μ g full length acid α -glucosidase cDNA cloned in the eukaryotic expression vector pSG5 (pSHAG2) (Green et al., 1988; Hoefsloot et al., 1990) was added to 1x10⁶ cells in 10 cm tissue culture dishes or 3 μ g pSHAG2 was added to 2x10⁵ cells

grown on coverslips in 3.5 cm tissue culture dishes, as a CaPi-precipitate. The plasmid pGA293 was used for the expression of E.coli β -galactosidase (mock) (An et al., 1982). After 16 hours the cells were treated with 15% glycerol for 1 minute at room temperature (Parker and Stark, 1979) and washed twice, once with plain medium and once with a saline solution that contained 1.0 mM EDTA, to dissolve any residual precipitate. The glycerol-shocked cells were incubated at 37°C in a 5% CO₂ atmosphere in complete medium until they were harvested for biochemical analysis or processed for immunofluorescence and immunoelectron microscopy.

Stably transfected MDCK-II cells expressing acid α -glucosidase were generated by transfecting cells with the pMAM neo blue vector (Clontech) containing full length acid α -glucosidase cDNA. Stably transfected LLC-PK₁ cells were generated by co-transfection with pSHAG2 and PITA (a plasmid conferring resistance to the antibiotic G418; in a molecular ration pSHAG2:PITA of 9:1). MDCK-II and LLC-PK₁ cells were transfected as described above. One day after the glycerol shock, MDCK II cells were split at a 1:10 dilution and LLC-PK₁ cells at a 1:5 dilution in culture medium containing 800 μ g/ml G418 (Southern and Berg, 1982). This medium was changed every fourth day. Colonies of drug resistant cells were visible after 8 days of growth under G418 selection and were transferred to multiwell culture plates (Costar 24 well plates) 3 to 4 days later. Colonies expressing acid α -glucosidase (screened by means of enzyme activity) were trypsinized and subcloned by limiting dilution in 96-well microtiter plates (Costar).

Biochemical Assays.

Cell lysates were prepared by sonication of cell pellets in distilled water. Protein concentrations of cell homogenates were measured with the use of the BCA protein assay kit (Pierce), using bovine serum albumin as standard. Acid α -glucosidase and β -hexosaminidase activities were determined with the artificial substrates 4-methylumbelliferyl- α -D-glucopyranoside and 4-methylumbelliferyl- β -D-N-acetylglucosaminide, respectively (Melford) as described by Galjaard, (1980).

Immunocytochemistry.

Transiently transfected cells on coverslips were prepared for immunocytochemistry as described before (Van Dongen et al., 1984). Incubations were performed with rabbit polyclonal antibodies against human acid α -glucosidase (Reuser et al., 1985). Immune complexes were visualized with goat anti-rabbit IgG conjugated to fluorescein.

For immuno-electronmicroscopy cells were fixed in 1% acrolein and 0.4% glutaraldehyde in 0.15 M sodium bicarbonate, pH 7.4, for 1 hour at 4°C when 10 cm dishes were used, or in a mixture of 0.1% glutaraldehyde and 1% formaldehyde in 0.15 M sodium bicarbonate, pH 7.4, for 1 hour at room temperature in case filter grown cells were used. After fixation, the cells were washed with PBS and gently scraped off the culture dish or the membrane filter with a rubber policeman. Cells were embedded in 10% gelatin. Ultrathin cryosections were immunostained by incubation with rabbit polyclonal antibodies, followed by an incubation with goat anti-(rabbit IgG) coupled to 10 nm colloidal gold (GAR-10; Aurion) as described before (Van Dongen et al., 1985).

Electron Microscopy.

For morphological studies filter-grown stably transfected cells were fixed on the filter with 4% paraformaldehyde and 1% glutaraldehyde in PBS, pH 7.3, for 16 hours at 4°C. After rinsing two times for 30 minutes in 0.1 M cacodylate buffer, pH 7.3, at 4°C, the cells were postfixed at 4°C in 0.1 M cacodylate buffer, pH 7.15 containing 1% OsO₄ and 50 mM K₂Fe(CN)₆. After rinsing in 0.1M cacodylate buffer, pH 7.3, at room temperature the cells were dehydrated in alcohol and embedded in a thin layer of epon. After polymerization, the material was cut into small pieces and embedded in epon again. Ultrathin sections were cut parallel to the long axis of the cells with a Reichert OMU3 ultramicrotome. These sections were stained with uranylacetate and lead citrate and viewed in a Philips EM 400 electronmicroscope at 80 kV.

Metabolic Labelling.

For studies on the biosynthesis of acid α -glucosidase, stably transfected MDCK-II cells grown on polycarbonate filters, were metabolically labelled with [³⁵S] methionine (1000 Ci/mmol) and [³H] leucine (120-190 Ci/mmol; Amersham). After preincubation for one hour at 37°C in leucine free medium followed by a two hours labelling period at 37°C with 100 μ Ci [³⁵S] methionine and 100 μ Ci [³H] leucine per filter (added to the basolateral side), the cells were washed with PBS and cultured for various periods in complete medium without radioactive precursor. The cells were then scraped off the filter and solubilized as described by Hasilik and Neufeld (1980a). Acid α -glucosidase was immunoprecipitated with affinity purified antibodies against human placental acid α -glucosidase (Reuser et al., 1985). Some samples were

deglycosylated with Endo H or Endo F according to the instructions of the manufacturer (Boehringer). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed on 8% slab gels according to Laemmli (1970). The radioactive bands were visualized by fluorography.

Triton X-114 phase separation.

For assessment of membrane association of acid α -glucosidase in stably transfected MDCK-II cells, the cells were harvested by scraping, and washed two times in PBS. Cells were lysed in a buffer containing 10 mM Tris/HCl (pH 7.4), 150 mM NaCl, and 0.6% Triton X-114 at 0°C. Proteins were separated in hydrophilic (water-phase) and membrane-associated (detergent-phase) fractions as described by Bordier (1981). Acid α -glucosidase was immunoprecipitated from the detergent-phase and the water-phase with the rabbit polyclonal antibody. Precipitated proteins were separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Laemmli, 1970), and blotted onto nitrocellulose (Towbin et al., 1979). Acid α -glucosidase was visualized using the rabbit polyclonal antibody in combination with [¹²⁵I] protein A as described by Reuser et al. (1987). Blots were exposed to Kodak XAR films.

Enzyme secretion assay.

Stably transfected cell lines were cultured to confluency on polycarbonate membranes (diameter 24.5 mm; 3 μ m pore size). The apical and basolateral compartments were filled with 2 ml of DMEM supplemented with 10% pH inactivated fetal calf serum and antibiotics. Medium samples were taken in a time course in order to determine enzyme activity.

Enzyme uptake.

Mannose 6-phosphate containing purified bovine testis acid α -glucosidase (Reuser et al., 1984) was added to confluent MDCK II and LLC-PK₁ cells on filters in an amount equivalent to an activity of 1 μ mol 4-methylumbelliferon/h/ml medium. The medium used for uptake studies was Ham's F10 medium supplemented with 10% fetal calf serum and antibiotics. Piperazine N,N'-bis-2-ethanesulfonic acid (Pipes, BDH Chemicals) was added in a concentration of 3 mM to maintain the pH between 6.8 and 7.0. Cells were incubated overnight at 37°C in an atmosphere of 5% CO₂ and 95% air. Cells were harvested, and homogenates were prepared by sonication in distilled water.

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Chapter 8

Biosynthesis, posttranslational modification and intracellular transport of sucrase-isomaltase compared to lysosomal acid α -glucosidase

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Summary

The biosynthesis and the intracellular transport of the intestinal brush border enzyme complex of sucrase-isomaltase and the structurally and functionally related lysosomal enzyme acid α -glucosidase were compared. COS-1 cells were transiently transfected with cDNA constructs coding for these enzymes to obtain high levels of expression. The localization of sucrase-isomaltase and acid α -glucosidase in the COS-1 cells was found to mimic the subcellular distribution of the enzymes in intestinal epithelial cells and in the colon carcinoma derived Caco-2 cell line. Sucrase-isomaltase was predominantly associated with the plasma membrane whereas acid α -glucosidase was mainly contained in lysosomes. A fraction of sucrase-isomaltase was routed to the lysosomes and a fraction of acid α -glucosidase to the plasma membrane. Lysosomal targeting of sucrase-isomaltase via the mannose 6-phosphate receptor was excluded by demonstration of the lack of the mannose 6-phosphate recognition marker.

In an attempt to specify protein domains involved in the preferential targeting of sucrase-isomaltase to the plasma membrane and acid α -glucosidase to the lysosomes, truncated forms of isomaltase were produced, and a fusion protein was constructed in which the 5' half of isomaltase was combined with 3' half of acid α -glucosidase. These artificial forms of isomaltase and acid α -glucosidase were, however, trapped in the endoplasmic reticulum when expressed in COS-1 cells. Models for the *ectopic* localization of sucrase-isomaltase and acid α -glucosidase are discussed.

Introduction

Sucrase-isomaltase (EC 3.2.1.48 & EC 3.2.1.10) occurs as a complex of two enzymes in the brush-border of intestinal epithelium. The sucrase and isomaltase subunits are derived from a single chain precursor which is cleaved when exposed to pancreatic proteases present in the intestinal lumen. The isomaltase subunit is anchored in the apical cell membrane via its hydrophobic amino-terminal segment (Brunner et al., 1979). This segment also serves as signal peptide to guide the precursor of sucrase-isomaltase co-translationally into the lumen of the endoplasmic reticulum. The sucrase subunit itself is not membrane anchored but remains associated with isomaltase via strong ionic interactions (Hunziker et al., 1986). The molecular mass of the uncleaved sucrase-isomaltase precursor is approximately 260 kDa including an estimated 15% of carbohydrate in the form of N-linked and O-linked oligosaccharide side chains (Cogoli et al., 1972; Hunziker et al., 1986; Naim et al., 1988b). The molecular mass of the sucrase and isomaltase subunits is 130 kDa and 145 kDa, respectively. Based on sequence analysis it is assumed that the sucrase-isomaltase gene has arisen by duplication of an ancestral gene (Hunziker et al., 1986). The 5' half of the gene contains the coding sequence for isomaltase whereas the information for sucrase is contained in the 3' half. The amino acid sequence homology of sucrase and isomaltase is 41% (Hunziker et al., 1986). Both sucrase and isomaltase are disaccharidases with an evolutionary conserved substrate specificity for maltose. Isomaltase has additional specificity for isomaltose and sucrase for sucrose.

An enzyme structurally related to sucrase and isomaltase is the lysosomal enzyme acid α -glucosidase (EC 3.2.1.3) (Hoefsloot et al., 1988). The homology between acid α -glucosidase and sucrase is 36% at the protein level. This figure is 40% when acid α -glucosidase is compared with isomaltase. Functional homology consists of substrate specificity of acid α -glucosidase for maltose and isomaltose. Acid α -glucosidase is unique in its glycogen degrading activity.

The precursor of acid α -glucosidase is N-linked glycosylated at each of the seven potential sites (Hermans et al., 1993) and has approximately the same molecular mass as the sucrase and isomaltase subunits (110 kDa). Phosphorylation of mannose residues enables the precursor to bind to the mannose 6-phosphate receptor as a means to reach the lysosomes, where proteolytic processing to 76 kDa and finally 70 kDa occurs (Hasilik and Neufeld, 1980a/b; Reuser et al., 1985, 1987).

Of further interest for the comparison of sucrase-isomaltase and acid α -glucosidase is the intracellular localization of these enzymes. A small but significant amount of sucrase-isomaltase seems to be routed to the lysosomes despite of sucrase-isomaltase being a typical plasma membrane enzyme complex (Fransen et al., 1985; Matter et al., 1990). At the same time, acid α -glucosidase occurs at the plasma membrane of certain cell types while being a

typical lysosomal enzyme (Fransen et al., 1988; Oude Elferink et al., 1989; Hoefsloot et al., 1990; Willemsen et al., 1991; Klumperman et al., 1991).

In this paper we report on the biosynthesis and intracellular transport of sucrase-isomaltase compared to acid α -glucosidase in transiently transfected COS-1 cells.

Results

The biosynthesis and posttranslational modification of sucrase-isomaltase was studied in transiently transfected COS-1 cells. A comparison was made with the structurally and functionally related lysosomal enzyme acid α -glucosidase. The activities of the respective enzymes were assayed at 72 hours after transfection. Table I shows the data of a representative experiment. Maltase activity (α -1,4) was measured with the artificial substrate 4-methylumbelliferyl- α -D-glucopyranoside, whereas the hydrolytic activity toward α -1,6 glycosidic linkages was determined with isomaltose as substrate. Sucrase activity was measured by the amount of glucose liberated from sucrose. A transfection with bacterial β -galactosidase cDNA was performed as measure for the endogenous enzyme activities. Cells transfected with sucrase-isomaltase cDNA were able to hydrolyse sucrose, and both α -1,4 and α -1,6 glycosidic linkages. The maltase activity was 27 times above the endogenous background. Endogenous isomaltase and sucrase activities were not detectable. COS-1 cells transfected with lysosomal α -glucosidase cDNA were expressing fifteen times higher acid α -glucosidase activity than β -galactosidase transfected cells. The isomaltase and sucrase activity of cells transfected with acid α -glucosidase cDNA was below detection.

The biosynthesis of sucrase-isomaltase and acid α -glucosidase was studied via pulse-chase labelling. Figure 1 shows that sucrase-isomaltase was synthesized as a precursor of approximately 240 kDa in a one hour pulse period (0 hour of chase). No change of molecular mass was observed during a 20 hour chase period. The acid α -glucosidase precursor synthesized during a one hour pulse period had a molecular mass of 110 kDa while a 95 kDa intermediate and a 76 kDa mature species became apparent after a subsequent chase period of 20 hours (fig. 1). A fraction of the 110 kDa acid α -glucosidase precursor pool was secreted into the culture medium whereas this was not the case for sucrase-isomaltase.

The intracellular localization of sucrase-isomaltase and acid α -glucosidase was visualized using immunocytochemistry. Figure 2A shows that sucrase-isomaltase was present at the plasma membrane whereas a typical lysosomal labelling was obtained for acid α -glucosidase (Fig. 2B). The distinct subcellular localization of the two enzymes was confirmed by immuno-electronmicroscopy. The electron micrograph of figure 3 illustrates the plasma

Table I. Activity of rabbit sucrase-isomaltase and human acid α -glucosidase in transiently transfected COS-1 cells measured 72 hours after transfection.

cDNA construct	α -glucosidase/ maltase* (α -1,4)	isomaltase** (α -1,6)	sucrase**	β -hexosa- minidase*
Rabbit sucrase- isomaltase	406	1708	558	741
Human acid α -glucosidase	229	N.D.	N.D.	650
E.coli β -galactosidase	15	N.D.	N.D.	645

* expressed as nmol MU/h per mg of protein

** expressed as nmol glucose/h per mg of protein

N.D. stands for not detectable

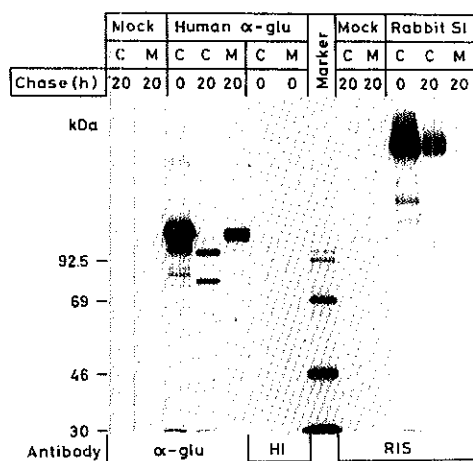


Figure 1. Synthesis and processing of human acid α -glucosidase and rabbit sucrase-isomaltase in COS-1 cells. COS-1 cells transfected with acid α -glucosidase and sucrase-isomaltase cDNA constructs were labelled for one hour with [3 H]-Leucine and harvested directly (0 hour chase) or after a subsequent period of 20 hours. Acid α -glucosidase and sucrase-isomaltase were immunoprecipitated with the antisera indicated from the cells (C) and the medium (M) and were analysed by SDS/PAGE. The molecular masses of marker proteins are indicated. As a negative control, COS-1 cells were transfected with a cDNA construct of E.coli β -galactosidase (Mock).

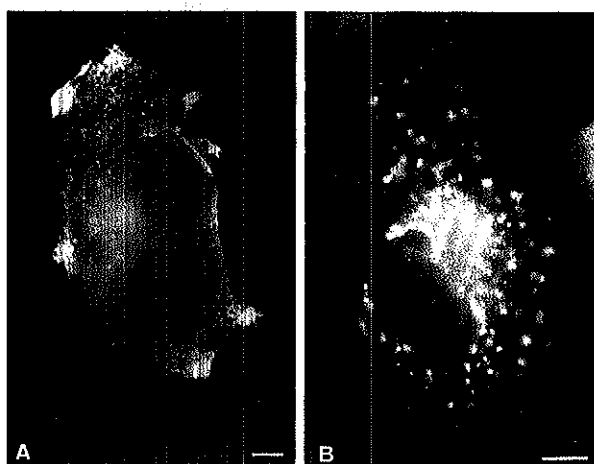
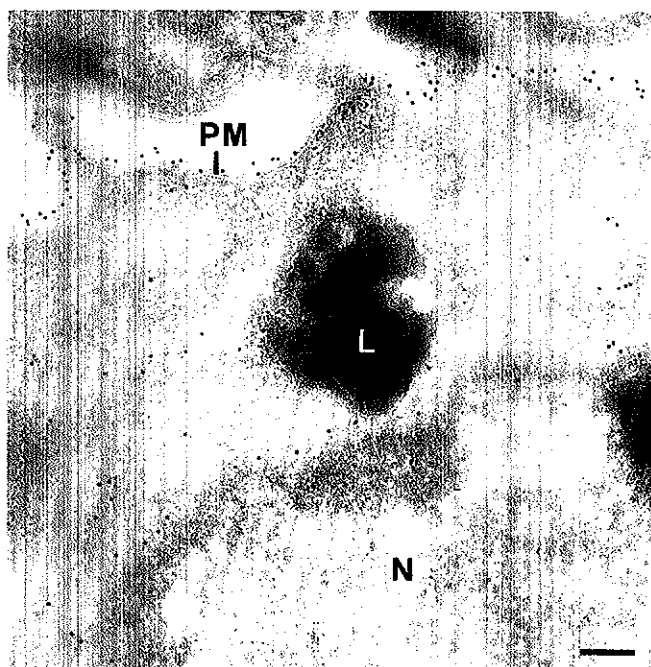


Figure 2. Localization of sucrase-isomaltase and acid α -glucosidase in transfected COS-1 cells. Transiently transfected cells were fixed and incubated with a guinea-pig antiserum against rabbit sucrase-isomaltase (A) or with a rabbit antiserum against human acid α -glucosidase (B). Immune complexes were visualized with goat anti-guinea pig or goat anti-rabbit IgG conjugated to fluorescein. Bar represents 10 μ m.

Figure 3. Intracellular localization of rabbit sucrase-isomaltase in transfected COS-1 cells. Ultrathin cryosections were prepared at 72 hours after transfection and incubated with a guinea-pig antiserum against rabbit sucrase-isomaltase followed by an incubation with rabbit anti-guinea pig and finally with protein A coupled to colloidal gold. Sucrase-isomaltase was associated with the nuclear envelope, the plasma membrane (PM) and the lysosome (L). Gold particles in the cytoplasm were associated with membranous structures probably representing cisternae of the rough endoplasmic reticulum. Bar represents 0.2 μ m.



membrane localization of sucrase-isomaltase. The figure also demonstrates that the nuclear envelope and lysosomes are labelled. The plasma membrane of untransfected cells was unlabelled (cell at top of Fig. 3). The lysosomal and plasma membrane localization of acid α -glucosidase in transiently transfected COS-1 cells was reported previously (Hoefsloot et al., 1990; Wisselaar et al., 1993).

To investigate whether the mannose 6-phosphate receptor could be instrumental in the lysosomal targeting of sucrase-isomaltase we studied the phosphorylation of this enzyme. Figure 4 compares the biosynthesis and phosphorylation of sucrase-isomaltase and acid α -glucosidase during a six hour labelling period. Phosphorylation of the 110 kDa precursor of acid α -glucosidase was evident but phosphorylation of sucrase-isomaltase could not be detected. Digestion of [32 P]-P_i labelled acid α -glucosidase with Endo H resulted in the loss of label indicating that the phosphate was linked to the oligosaccharides (data not shown).

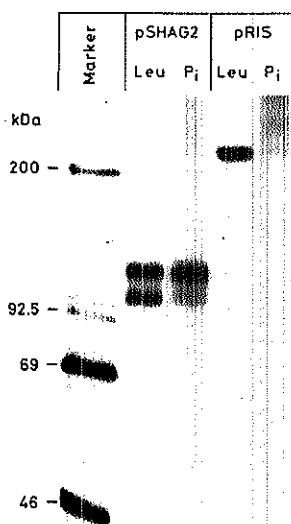


Figure 4. Biosynthesis and phosphorylation of acid α -glucosidase and sucrase-isomaltase in COS-1 cells. COS-1 cells transfected with acid α -glucosidase (pSHAG2) and sucrase-isomaltase (pRIS) cDNA constructs were labelled for six hours with [3 H]-Leucine or [32 P]-P_i. The proteins were analyzed as described in the legend of figure 1.

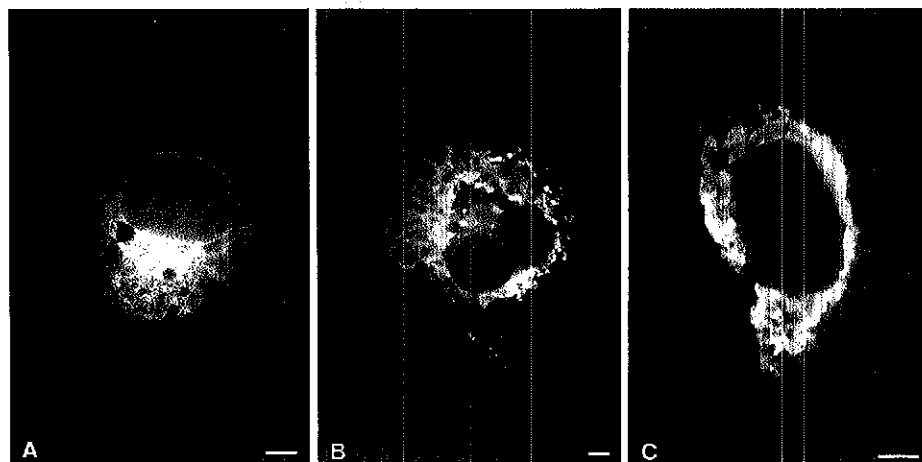


Figure 5. Localization of the human isomaltase/acid α -glucosidase fusion protein and of isomaltase in COS-1 cells. Immunofluorescence microscopy was performed as described in the legend of figure 2. The fusion protein (A), truncated rabbit isomaltase (B) and the truncated human isomaltase (C) were all located in a perinuclear region of the cell. Bar represents 10 μ m.

In an attempt to specify protein domains involved in the preferential routing of sucrase-isomaltase to the plasma membrane and acid α -glucosidase to the lysosomes, truncated constructs of isomaltase, and a fusion protein of isomaltase combined with acid α -glucosidase were made. The localization of these constructs expressed in COS-1 cells is illustrated in figure 5. In figure 5A the 481 amino acid residues long amino-terminal fragment of α -glucosidase was substituted by the homologous fragment of isomaltase. The picture shows a diffuse network of structures spreading from the nucleus into the cytoplasm which is typical for localization of a protein in the endoplasmic reticulum. Expression of the truncated forms of rabbit and human isomaltase resulted in a similar labelling pattern (Fig. 5B and 5C). Using immuno-electronmicroscopy, the single isomaltase subunits and the fusion protein appeared to be present in the endoplasmic reticulum but they were absent from the more distal compartments of the glycoprotein transport pathway (Fig. 6 and 7). The very weak Golgi labelling seen in figure 6B was also obtained in untransfected cells.

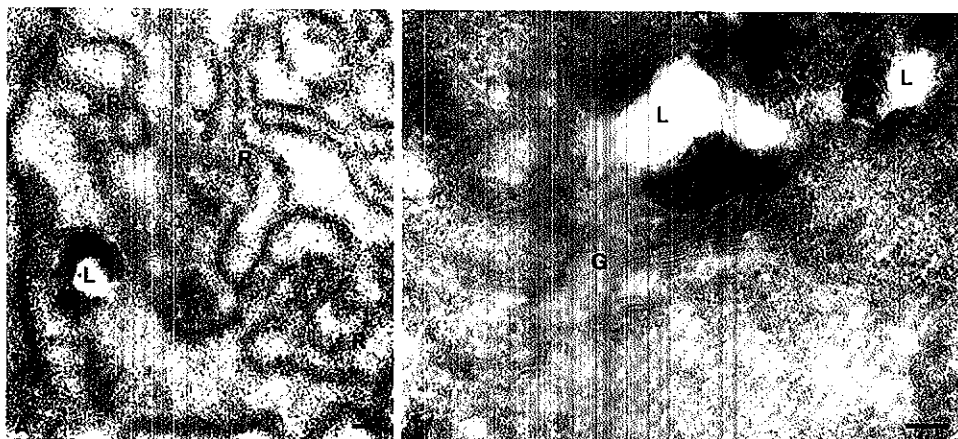


Figure 6. Intracellular localization of the human isomaltase/acid α -glucosidase fusion protein studied by immuno-electronmicroscopy. Ultrathin cryosections were incubated with rabbit antiserum against human acid α -glucosidase and subsequently with goat anti-rabbit IgG coupled to colloidal gold. The fusion protein was restricted in its localization to the rough endoplasmic reticulum (R). The Golgi complex (G) and the lysosomes (L) were not labelled. Bar represents 0.2 μ m.

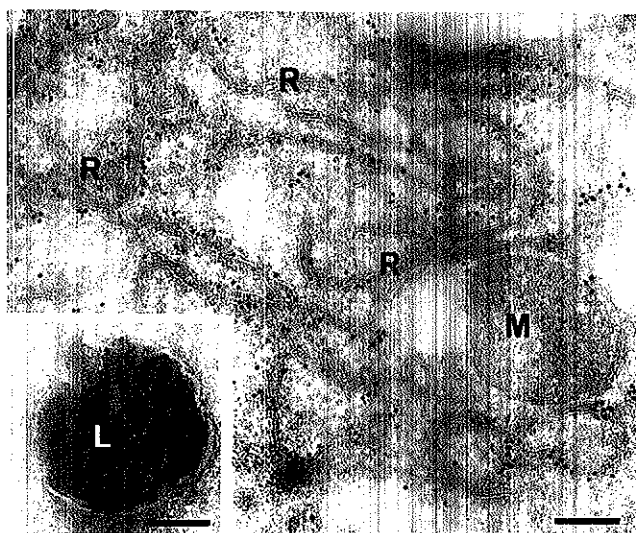


Figure 7. Intracellular localization of truncated human isomaltase in COS-1 cells. The procedure was as described in the legend of figure 6 except that antiserum against human isomaltase was used in the first incubation. The rough endoplasmic reticulum (R) was labelled, but the lysosomes (L) were unlabelled. Bar represents 0.2 μ m.

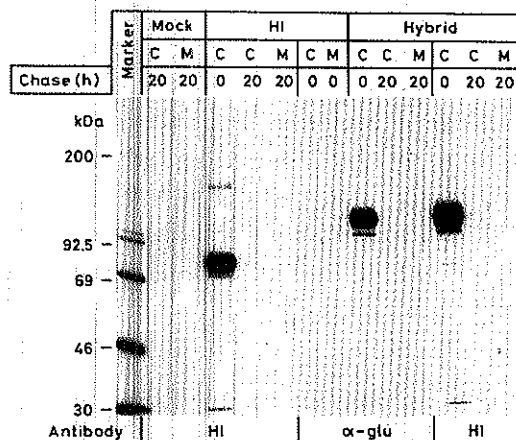


Figure 8. Biosynthesis of truncated human isomaltase and a human isomaltase/acid α -glucosidase fusion protein in COS-1 cells. Labelling and immunoprecipitation from cells (C) and medium (M) was carried out as described in the legend of figure 1.

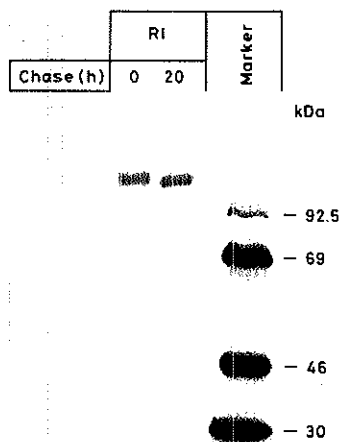


Figure 9. Synthesis of truncated rabbit isomaltase in COS-1 cells. The procedure was as described in the legend of figure 1.

The fate of the fusion protein and the single isomaltase subunits was studied via pulse-chase labelling (Fig. 8 and 9). The truncated human isomaltase subunit (HI) with a molecular mass of 76 kDa was detectable with the corresponding antibody (HI) after a pulse period of 1 hour (0 hour of chase). The protein was, however, degraded in the following 20 hours of chase. The human isomaltase/human acid α -glucosidase fusion protein with a molecular mass of 110 kDa was also subjected to degradation in the 20 hours of chase. The hybrid character of the fusion protein was demonstrated by its cross reaction with both antibodies raised against both acid α -glucosidase (α -glu) as well as human isomaltase. Only the rabbit isomaltase subunit of 110 kDa appeared to be stable during the chase period. None of the proteins was secreted into the medium and none was catalytically active for any of the natural or artificial substrates.

Discussion

Full length cDNA constructs of rabbit sucrase-isomaltase and human lysosomal acid α -glucosidase were transiently expressed in COS-1 cells to compare the differential routing of the two enzymes. The localization of the two enzymes in the COS-1 cells appeared to mimic the situation in intestinal epithelial cells and in the colon carcinoma derived Caco-2 cell line (Fransen et al., 1985, 1988; Matter et al., 1990; Klumperman et al., 1991). The major fraction of sucrase-isomaltase was associated with the plasma membrane, but enzyme was also present along the intracellular transport pathway including the endoplasmic reticulum, the Golgi complex and the trans Golgi network. A significant fraction of sucrase-isomaltase was found in the endocytic system of which the multivesicular bodies were most abundantly labelled. Also lysosomes contained sucrase-isomaltase.

The structurally and functionally related lysosomal enzyme acid α -glucosidase was demonstrated earlier to be localized in essentially the same compartments in intestinal epithelial cells, Caco-2 cells and transfected COS-1 cells (Fransen et al., 1988; Klumperman et al., 1991; Hoefsloot et al., 1990). In contrast to sucrase-isomaltase, however, the major fraction of acid α -glucosidase was contained in lysosomes and only a minor fraction was associated with the plasma membrane.

Both the lysosomal localization of sucrase-isomaltase and the plasma membrane localization of acid α -glucosidase seem of little functional significance. If sucrase-isomaltase would be an essential lysosomal enzyme one would expect the enzyme to be expressed in all different cell types. Sucrase-isomaltase expression is limited, however, to intestinal epithelial cells. An important function for acid α -glucosidase in the intestinal lumen cannot be envisaged either since the carbohydrates in the food are fully digested by the amylases and glucoamylases along the digestive tract.

Missorting of sucrase-isomaltase and acid α -glucosidase seems a more plausible explanation for the "ectopic" localization of both enzymes. It was demonstrated by Matter et al. (1990) that at least a fraction of the lysosomal sucrase-isomaltase in the Caco-2 cells was derived from the plasma membrane via endocytosis. A direct route from the trans Golgi network to the lysosomes was, however, assumed to account for the major fraction of sucrase-isomaltase in the lysosomes (Matter et al., 1990). This route would not be signal mediated but be a diversion from the main, signal mediated, pathway to the plasma membrane. It was proposed that the function of this pathway could be the regulation of the expression of sucrase-isomaltase in the intestine. There is no reason, however, to expect that a similar regulating mechanism would be functional in COS-1 cells.

Lysosomal targeting of sucrase-isomaltase via the mannose 6-phosphate receptor system can be excluded since we have shown that sucrase-isomaltase is not a substrate for N-acetylglucosamine phosphotransferase (Fig. 4). It therefore seems that the lysosomal fraction of sucrase-isomaltase originates from missorting in the trans Golgi network whereby it is channelled to the late endosomes. In this latter compartment it is likely to intermingle with the integral lysosomal membrane proteins and the membrane bound acid phosphatase and to accompany these enzymes to the lysosomes (Waheed et al., 1988; Braun et al., 1989; Fujita et al., 1991).

The signal responsible for targeting of sucrase-isomaltase to the plasma membrane is the membrane anchor formed by the uncleaved signal peptide. The signal for apical as opposed to basolateral sorting of sucrase-isomaltase in polarized (intestinal) epithelial cells is as yet unknown. The structural homology of sucrase-isomaltase and acid α -glucosidase does not extend to the signal peptide. In contrast to sucrase-isomaltase the signal peptide of acid α -glucosidase is predicted to be cleavable. We have demonstrated, however, that the precursor of acid α -glucosidase remains initially attached to the membrane via its signal peptide (Wisselaar et al., 1993). In analogy to sucrase-isomaltase it was proposed that a fraction of membrane bound acid α -glucosidase would be transported to the plasma membrane rather than to the lysosomes. From the plasma membrane the enzyme could ultimately reach the lysosomes via endocytosis. Phosphorylation of the acid α -glucosidase precursor at mannose residues opens the possibility for transport of soluble forms of acid α -glucosidase to the lysosomes via the mannose 6-phosphate receptors. The contribution of each of the available transport pathways is unknown and may vary by the conditions in particular cell types. In I-cells for instance, the activity of acid α -glucosidase can be close to normal despite the deficiency of N-acetylglucosamine phosphotransferase causing the complete deficiency of other soluble lysosomal enzymes that are transported via the mannose 6-phosphate receptor (Tsuji and Suzuki, 1988; Van Dongen et al., 1985).

The structural and conformational requirements for the phosphorylation of lysosomal enzymes has been a topic of interest. The most recent information is that lysosomal enzymes

may have more than one recognition domain for the N-acetylglucosamine phosphotransferase (Cantor and Kornfeld, 1992a). Each domain seems to effectuate with preference the phosphorylation of carbohydrate chains located in the same domain, but those located in other domains can be phosphorylated as well (Cantor and Kornfeld, 1992a). Flexibility of carbohydrate side chain phosphorylation was demonstrated by the addition and deletion of glycosylation sites in arylsulfatase A and in cathepsin D (Gieselmann et al., 1992; Cantor and Kornfeld, 1992b).

A fusion protein of human isomaltase and human acid α -glucosidase was produced to obtain a clue to the location of the N-acetylglucosamine phosphotransferase recognition domain of acid α -glucosidase. Phosphorylation of acid α -glucosidase was lost by substitution of the amino-terminal half of acid α -glucosidase by the amino-terminal half of isomaltase. It was however shown by immuno-light and electronmicroscopy that loss of phosphorylation was not due to loss of recognition but to the retention of the fusion protein in the endoplasmic reticulum. This effect was also seen for certain mutants of acid α -glucosidase and sucrase-isomaltase and is probably due to misfolding (Naim et al., 1988a; Hermans et al., 1991a, 1993; Klausner et al., 1990; Klausner and Sitia, 1990). Also the transport of truncated forms of human and rabbit isomaltase appeared to be blocked in the endoplasmic reticulum. The structures of sucrase, isomaltase and acid α -glucosidase are apparently too different to allow exchange of protein domains without interfering dramatically with transport. More subtle changes in the structures of the enzymes are required. For instance, it would be of interest to study in greater detail the significance of the signal peptide in the targeting of sucrase-isomaltase and acid α -glucosidase in polarized and non-polarized cells. The recent publication by Hegner et al. (1992) showing that a single amino acid substitution can convert the uncleaved signal peptide of sucrase-isomaltase to a cleavable signal sequence indicates that this approach should be feasible.

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Materials and methods

cDNA Constructs.

The human acid α -glucosidase cDNA (pSHAG2) (Hoefsloot et al., 1988), the rabbit sucrase-isomaltase cDNA (pRIS) (Hunziker et al., 1986) and the 2.076 kb fragment of human isomaltase (Green et al., 1987) were cloned in the eukaryotic expression vector pSG5 as described previously (Hoefsloot et al., 1988; Hermans et al., 1991b). Rabbit isomaltase cDNA was generated by digestion of rabbit sucrase-isomaltase cDNA with the

restriction enzymes EcoRI and BamHI. EcoRI cuts in the 5'polylinker and BamHI at nucleotide 2073 of the published rabbit sucrase-isomaltase cDNA sequence. The EcoRI-BamHI fragment was cloned in pSG5. To construct a fusion protein of 5'human isomaltase and 3'human acid α -glucosidase a Sma-I restriction site was introduced at position 1445 of the human isomaltase cDNA using site-directed mutagenesis as described by Hermans et al. (1991b). The acid α -glucosidase cDNA was subsequently cut with Sma-I at position 1663 and the 3' half was ligated into the Sma-I site of isomaltase at position 1445. The cDNA construct of human isomaltase/human acid α -glucosidase was cloned in the EcoRI site of pSG5.

Transfection procedures.

Monkey kidney COS-1 cells were cultured in Dulbecco's modified Eagle's medium, supplemented with antibiotics and 10% fetal calf serum, under air/CO₂ (9:1). COS-1 cells were transfected using the DEAE-dextran method exactly as described by Hoefsloot et al., 1990. Transfection with bacterial β -galactosidase cDNA (pGA 293, An et al., 1982) served as a negative control.

Biochemical assays.

Cell lysates were prepared by repeated freezing and thawing of cell pellets in distilled water. Protein concentrations of cell homogenates were measured with the use of a bicinchoninic acid (BCA) protein assay kit (Pierce) using bovine serum albumin as standard. Acid α -glucosidase and maltase activities were determined with the artificial substrate 4-methylumbelliferyl- α -D-glucopyranoside (Melford) at pH 4.3 and pH 5.6, respectively. Sucrase and isomaltase activities were determined with sucrose and isomaltose as the respective substrates at pH 5.6. The amount of liberated glucose was determined according to Koster et al., 1972. β -Hexosaminidase activity was measured with 4-methylumbelliferyl- β -D-N-acetylglucosaminide (Galjaard et al., 1973).

Immunocytochemistry.

For application of lightmicroscopy, the cells were seeded in low density on coverslips on the evening before analysis and prepared for immunocytochemistry as described (Van Dongen et al., 1984). Incubations were performed with rabbit antiserum against human acid α -glucosidase (Reuser et al., 1985); rabbit antiserum against human isomaltase (3/705/60; Green et al., 1987) and with guinea-pig antiserum against rabbit sucrase-isomaltase (gp 71; kindly provided by Dr. H. Wacker). Immune complexes were visualized with goat anti-rabbit and with goat anti-guinea pig IgG conjugated to fluorescein.

For immuno-electronmicroscopy cells were fixed in 1% acrolein and 0.4% glutaraldehyde in 0.15 M sodium bicarbonate (pH 7.4) for 1 hour at 4°C. The cells were washed with phosphate buffered saline and gently scrapped off the culture dish with a rubber policeman. Cells were embedded in 10% gelatin. Ultrathin cryosections of acid α -glucosidase and human isomaltase transfected cells were immunostained by incubation with the antiserum described above followed by an incubation with goat anti-(rabbit IgG) coupled to 10 nm colloidal gold (GAR-10, Aurion). Rabbit sucrase isomaltase was visualized by an indirect immunostaining method. Ultrathin sections were incubated first with guinea-pig antiserum against rabbit sucrase-isomaltase followed by an incubation with rabbit anti-guinea pig antiserum and a final incubation with protein A coupled to 10 nm colloidal gold (Aurion). Control sections were incubated with normal rabbit serum instead of specific antiserum.

Metabolic labelling.

Transiently transfected COS-1 cells were metabolically labelled with [³H] leucine or with [³²P] phosphate (Amersham) as described (Hasilik and Neufeld, 1980a,b). The cells were pulse labelled with [³H] leucine for one hour and harvested directly or after a subsequent chase of 18 hours. Cells labelled with [³²P] phosphate were harvested after 6 hours. Acid α -glucosidase, sucrase and isomaltase were immunoprecipitated with the respective antiserum described above. Some samples were digested with Endo H according to the instructions of the manufacturer (Boehringer). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed on 8% slab gels according to Laemmli (1970). The radioactive bands were visualized by fluorography.

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Summary

The mechanism of intracellular protein transport has attracted considerable attention in the past decade. A number of sorting signals imprinted in the protein structure have been characterized. Receptors have been identified in addition, which recognize these sorting signals. It has also become apparent that the mechanism of protein transport can differ per cell type and that transport of proteins is accompanied by posttranslational modification (chapter 1).

The experimental work described in this thesis adds to the existing knowledge about the posttranslational modification and the mechanism of transport of two enzymes that are structurally and functionally related. These enzymes are acid α -glucosidase and the enzyme complex of sucrase and isomaltase. The first enzyme is responsible for the degradation of glycogen in the lysosomes, whereas the second is localized in the epithelium of the small intestine and involved in the hydrolysis of maltose and sucrose (chapter 2).

Mutations in the gene coding for acid α -glucosidase can give rise to deficiency of this enzyme and can thereby cause the inherited lysosomal glycogen storage disease type II. These mutations appear to interfere often with the intracellular transport of acid α -glucosidase and they may result in aberrant or incomplete posttranslational modification. In case of sucrase-isomaltase deficiency five different abnormalities are distinguished in the literature, depending on whether the mutation affects the transport, the posttranslational modification or the function of the enzyme complex (chapter 2).

In this thesis the intracellular transport and the posttranslational modifications of acid α -glucosidase and sucrase-isomaltase have been studied by using a cell free mRNA translation system or alternatively by using mammalian cells in which the enzymes are expressed via transfection of cDNA (chapter 4,6,7 and 8). The natural biosynthesis of acid α -glucosidase was studied in liver cells, and in fibroblasts (chapter 5).

Acid α -glucosidase is equipped with an amino-terminal signal peptide that acts as a first sorting signal. In chapter 4 it is described that acid α -glucosidase remains initially attached to the membrane of the endoplasmic reticulum via this signal peptide. Seven sugar chains are coupled to acid α -glucosidase while the enzyme resides in the endoplasmic reticulum (the sites of sugar chain attachment are indicated by the tripeptide Asn-X-Thr/Ser). This was demonstrated by individual elimination of each of the seven sites (chapter 6). The sugar chains are subjected to several modifications while the enzyme is transported from the endoplasmic reticulum

through the Golgi complex to the lysosomes. The importance of glycosylation is illustrated by the fact that transport of acid α -glucosidase is blocked and posttranslational modifications do not occur when glycosylation is inhibited completely. Under these circumstances the protein is degraded (chapter 4). When only the second glycosylation site, at Asn 233, is eliminated the acid α -glucosidase precursor accumulates in the endoplasmic reticulum. Individual elimination of the other six sites does not have a significant effect on transport or function (chapter 6). The post-translational modification of acid α -glucosidase is delayed when the sugar modifying enzymes in the endoplasmic reticulum are inhibited (chapter 5).

Sugar modifications can be essential for recognition of a protein by a receptor. For instance the phosphorylation of selected mannose residues is essential for the binding of lysosomal enzymes to the mannose 6-phosphate receptor (MPR). This receptor is instrumental in the sorting and transport of lysosomal enzymes to the lysosomes. For acid α -glucosidase it was demonstrated that at least two of the seven sugar chains become phosphorylated (chapter 6), and furthermore, that those sugar chains that do not obtain the mannose 6-phosphate group are converted from high mannose to complex type of chains. The extent of complex glycosylation depends on the time of sojourn in the Golgi complex (chapter 4).

Lysosome directed transport of acid α -glucosidase can thus be mediated via the MPR, but the membrane bound character of the acid α -glucosidase precursor implies the possible use of a MPR independent transport route to the lysosome. This independent route can lead directly from Golgi complex to lysosomes or it may involve transport via the plasma membrane. The presence of acid α -glucosidase on the plasma membrane of certain cell types, as was demonstrated by immuno-electron microscopy, suggests that at least a part of the newly synthesized molecules follow the indirect pathway. Acid α -glucosidase sub-species that are released from their membrane anchor but not bound by the MPR are secreted (chapter 4).

The modifications that occur after exit of acid α -glucosidase from the Golgi complex involve mainly the proteolytic removal of amino- and carboxyl-terminal peptides. Thiol proteinases are involved in this process. Maturation is of functional importance, since it increases the specificity of the enzyme for its natural substrate glycogen (chapter 4).

Chapter 7 deals in more detail with the transport of acid α -glucosidase in polarized cells. These are cells with structurally distinct and separated apical and basolateral plasma membrane domains. Transfected kidney tubule cells with an optimal degree of polarization were used as model system. These cells appeared to secrete a significant amount of acid α -glucosidase with strong preference from the apical cell surface. An actual association of acid α -glucosidase with the apical cell

membrane as observed in intestinal epithelial cells was not observed. In a model describing the polarized transport and secretion of acid α -glucosidase in kidney epithelial cells it is assumed that the apical sorting occurs by default (chapter 3 and 7). It is considered unlikely that specific apical sorting signals are imprinted in the primary structure of acid α -glucosidase. The apical membrane association of acid α -glucosidase in intestinal epithelial cells can be explained by anchorage of the enzyme via its uncleaved signal peptide when it is taken into consideration that acid α -glucosidase has strong structural similarity with the typical intestinal enzyme complex of sucrase and isomaltase which is also targeted to the apical cell surface.

Also the sucrase-isomaltase enzyme complex is synthesized with an amino-terminal signal peptide which functions as membrane anchor, but the anchorage is permanent. Sucrase-isomaltase is a glycoprotein like acid α -glucosidase. The human enzyme and the enzyme of rabbits have 18 and 19 potential sites for asparagine (Asn) linked glycosylation. However, in contrast to acid α -glucosidase none of the sugar chains becomes phosphorylated. As a consequence transport to the lysosomes via the MPR is excluded (chapter 8). Sucrase-isomaltase is not subjected to proteolytic processing during intracellular transport, despite the high degree of homology of acid α -glucosidase and sucrase-isomaltase. The structural differences are apparently of such nature that the intracellular transport and the posttranslational modification of both enzymes is significantly different. Protein domains can not be exchanged without a dramatic effect (chapter 8).

Sucrase-isomaltase is transported mainly to the plasma membrane in transfected monkey kidney cells. A portion of the newly synthesized enzyme reaches the lysosomes, but it is as yet unknown whether the enzyme is transported directly from the trans Golgi network to the lysosomes, or whether it reaches the lysosomes indirectly via the plasma membrane. The presence of sucrase-isomaltase in multi-vesicular bodies (endosomes) can be taken as indication for indirect transport, but can also point to continuous exchange of enzyme molecules between the plasma membrane and endosomes.

The cumulated data on the function, the synthesis and the transport-associated posttranslational modifications of acid α -glucosidase provide a solid basis for the interpretation of aberrant enzyme function in the lysosomal glycogen storage disease type II.

Samenvatting

Het mechanisme van intracellulair eiwit transport staat de laatste jaren sterk in de belangstelling. Diverse "sorteer signalen", ingebouwd in de structuur van eiwitten, zijn inmiddels gekarakteriseerd. Ook zijn receptoren geïdentificeerd die deze sorteer signalen herkennen (hoofdstuk 1). Tevens is duidelijk geworden dat het transport mechanisme per cel type kan verschillen en dat transport van eiwitten vaak gepaard gaat met posttranslationele modificaties.

Het experimentele werk beschreven in dit proefschrift is een aanvulling op de bestaande kennis over de posttranslationele modificaties en de transport mechanismen van twee structureel en functioneel verwante enzymen. Deze enzymen zijn het "zure" α -glucosidase, verantwoordelijk voor glycogeen afbraak in het lysosoom, en sucrase-isomaltase, een enzym complex dat in het dunne darmepitheel voorkomt en betrokken is bij de splitsing van (iso-) maltose en sucrose (hoofdstuk 2).

Mutaties in het gen coderend voor zure α -glucosidase kunnen leiden tot een deficiëntie van dit enzym en daarmee de erfelijke lysosomale stapelingsziekte glycogenose type II veroorzaken. Dergelijke mutaties interfereren vaak met het intracellulaire transport van zure α -glucosidase en resulteren in foutieve of onvolledige posttranslationele modificatie. Bij deficiëntie van sucrase-isomaltase worden in de literatuur vijf verschillende abnormaliteiten onderscheiden al naar gelang een defect te constateren is bij het transport, de posttranslationele modificatie dan wel de functie van het enzym complex (hoofdstuk 2).

Het intracellulaire transport en de posttranslationele veranderingen van zure α -glucosidase en sucrase-isomaltase zijn in dit proefschrift bestudeerd door gebruik te maken van een celvrij mRNA translatie systeem en van zoogdier cellen waarin deze enzymen via transfectie van cDNA tot expressie werden gebracht (hoofdstuk 4,6,7 en 8). Daarnaast werd de natuurlijke biosynthese van zure α -glucosidase in lever cellen en in fibroblasten bestudeerd (hoofdstuk 5).

Zure α -glucosidase heeft evenals andere lysosomale, secretie- en plasma membraan glycoproteïnen een amino-terminaal "signaal peptide" dat als sorteer signaal functioneert. In hoofdstuk 4 wordt beschreven dat zure α -glucosidase via deze signaal peptide sequentie een tijd lang verankerd blijft aan de membraan van het endoplasmatisch reticulum en mogelijk het Golgi complex, waardoor het zich gedraagt als een type II trans-membraan eiwit. Aan deze precursor van zure α -glucosidase worden in het endoplasmatisch reticulum zeven suiker ketens gekoppeld op plaatsen die aangegeven worden door de aminozuur volgorde Asn-X-Thr/Ser. Dit werd aangetoond door de zeven plaatsen individueel te deleteren en vervolgens het

effect te bestuderen (hoofdstuk 6). Tijdens transport door het endoplasmatisch reticulum en het Golgi complex ondergaan de suiker ketens, die aanvankelijk voornamelijk zijn opgebouwd uit mannose residuen, diverse veranderingen die de stabiliteit, het transport en de maturatie van zure α -glucosidase sterk kunnen beïnvloeden. Het belang van suiker ketens wordt onder andere geïllustreerd door het feit dat zure α -glucosidase niet wordt getransporteerd, geen posttranslationele veranderingen ondergaat en wordt afgebroken wanneer de glycosylering totaal wordt verhinderd (hoofdstuk 4). Bij eliminatie van alleen de tweede glycosylerings plaats (Asn 233), via gerichte mutagenese, werd ophoping van zure α -glucosidase in het endoplasmatisch reticulum waargenomen (hoofdstuk 6). Geen effect werd waargenomen wanneer de overige zes ketens ieder afzonderlijk verwijderd werden. Verder bleek het proces van posttranslationele modificatie van zure α -glucosidase vertraagd te worden bij remming van de suiker modificerende enzymen uit het endoplasmatisch reticulum (hoofdstuk 5).

Suiker modificaties kunnen van essentieel belang zijn voor herkenning van een eiwit door een receptor. Zo is de fosforylering van bepaalde mannose groepen essentieel voor de binding van lysosomale enzymen aan de mannose 6-fosfaat receptor (MPR), die zorgt voor sortering en transport naar het lysosoom. Wat betreft zure α -glucosidase werd door nader onderzoek aangetoond dat tenminste twee van de zeven suiker ketens gefosforyleerd worden (hoofdstuk 6) en dat een deel van de niet gefosforyleerde ketens wordt omgebouwd van ketens met veel mannose residuen tot zogenaamde complexe suiker ketens. De mate waarin dit gebeurt is gecorreleerd aan de tijd die het eiwit in het Golgi complex doorbrengt (hoofdstuk 4).

Lysosomaal transport van zure α -glucosidase kan dus verlopen via de MPR, maar het membraan gebonden karakter van de precursor impliceert dat er ook een MPR onafhankelijke transport route naar het lysosoom mogelijk is. Deze MPR onafhankelijke route kan direct van Golgi complex naar lysosoom leiden of indirect via de plasma membraan lopen. De aanwezigheid van α -glucosidase op de plasma membraan van bepaalde cel typen, zoals aangetoond met behulp van immunoelectronen microscopie, suggereert dat een deel van de moleculen de indirecte weg volgt. Daarnaast zijn er zure α -glucosidase sub-species die noch het lysosoom, noch de plasma membraan bereiken maar worden uitgescheiden omdat ze kennelijk worden los geknipt van het amino-terminale signaal peptide maar niet aan de MPR binden (hoofdstuk 4).

De modificaties die optreden nadat zure α -glucosidase het Golgi complex heeft verlaten betreffen voornamelijk de afsplitsing van peptiden aan de amino- en carboxyl uiteinden van het eiwit. Bij dit maturatie proces zijn thiol proteinases betrokken. Maturatie is van functioneel belang, omdat zure α -glucosidase er een

optimale specificiteit voor glycogeen door verkrijgt (hoofdstuk 4).

In hoofdstuk 7 wordt nader ingegaan op het transport van zure α -glucosidase in gepolariseerde cellen, dat zijn cellen met een structureel verschillend en gescheiden apicaal en basolateraal plasma membraan domein. Getransfecteerde nier tubuli cellen met een optimale graad van polarisatie werden als model systeem gebruikt. In deze cellen bleek een aanzienlijke hoeveelheid zure α -glucosidase niet naar het lysosoom getransporteerd te worden, maar gesecreteerd te worden. Daarbij was er een sterke preferentie voor secretie van het apicale cel oppervlak. Anders dan in darmepitheel cellen kon associatie van zure α -glucosidase met het apicale cel oppervlak niet worden vastgesteld. Het model dat naar aanleiding van deze studies werd opgesteld suggereert dat de apicale secretie / transport route de voorkeurs route (default route) is in gepolariseerde nier epitheel cellen en dat basolateraal transport gemedieerd wordt door sorteer signalen die kennelijk in zure α -glucosidase ontbreken (hoofdstuk 3 en 7). De apicale membraan associatie van zure α -glucosidase in darm epitheel cellen kan verklaard worden door verankering via de signaal peptide, als aangenomen wordt dat zure α -glucosidase op grond van fysisch-chemische eigenschappen dezelfde transport route volgt als het typische darm enzym complex sucrase-isomaltase, dat qua structuur sterk lijkt op zure α -glucosidase (hoofdstuk 7).

Ook sucrase-isomaltase heeft een amino-terminaal signaal peptide dat als membraan anker functioneert, maar de verankering is permanent. Evenals zure α -glucosidase is sucrase-isomaltase een glycoproteïne. Het enzym van de mens en het konijn heeft 18 respectievelijk 19 potentiële koppelings plaatsen voor asparagine (Asn) gebonden suiker ketens. Geen van de suiker ketens wordt echter gefosforyleerd, zodat transport naar het lysosoom via de MPR niet mogelijk is (hoofdstuk 8). Van sucrase-isomaltase worden geen fragmenten aan de amino- en carboxyl uiteinden afgesplitst gedurende het intracellulaire transport. Ondanks de hoge mate van homologie van sucrase-isomaltase en zure α -glucosidase zijn de structurele verschillen van beide eiwitten kennelijk van een dusdanige aard dat het intracellulaire transport en de post-translationele modificaties significant anders verloopt. Eiwit domeinen kunnen ook niet zonder meer uitgewisseld worden (hoofdstuk 8). In getransfecteerde apenier cellen wordt sucrase-isomaltase voornamelijk naar de plasma membraan getransporteerd. Een klein deel bereikt de lysosomen. Het is vooralsnog onduidelijk of dit deel direct vanaf het trans Golgi netwerk of indirect via de plasma membraan naar het lysosoom getransporteerd wordt. De aanwezigheid van sucrase-isomaltase in multivesiculaire bodies (endosomen) wekt de indruk van indirect transport maar kan ook duiden op continue uitwisseling van enzym tussen de plasma membraan en de endosomen.

De verzamelde gegevens over de functie, de synthese en de transport gekoppelde post-translationele verandering van zure α -glucosidase dienen als basis voor de interpretatie van afwijkende enzym functie bij de lysosomale glycogeen stapelings ziekte type II.

Curriculum Vitae

1 september 1962	Geboren te Capelle aan den IJssel
1974 - 1978	M.A.V.O.
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15 juli 1987	Aanvang promotie-onderzoek op de afdeling Celbiologie en Genetica bij Prof. Dr. H. Galjaard en Dr. A.J.J. Reuser, Erasmus Universiteit Rotterdam.
2 maart 1992	Aanvang als wetenschappelijk medewerkster op de afdeling Inwendige Geneeskunde III bij Prof. Dr. S. Lamberts, Erasmus Universiteit Rotterdam.

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Nawoord

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Stellingen behorende bij het proefschrift

**POSTTRANSLATIONAL MODIFICATIONS AND
INTRACELLULAR TRANSPORT OF LYSOSOMAL
 α -GLUCOSIDASE AND SUCRASE-ISOMALTASE**

Rotterdam, 10 maart 1993

Heleen A. Wisselaar

I

De precursor van het lysosomale enzym α -glucosidase is een type II transmembraan eiwit.

II

De mate van "complexe" glycosylering van glycoproteïnen wordt mede bepaald door de verblijftijd in het Golgi complex.

III

Eiwitten zonder specifieke transport signalen kunnen in MDCK-II cellen zowel naar de apicale als naar de basolaterale plasma membraan worden getransporteerd.

IV

Afgaande op het totale aantal lysosomale enzymen en de onderlinge verschillen in hun synthese snelheid is het onlogisch te veronderstellen dat overexpressie van één van de lysosomale enzymen tot een tekort aan mannose 6-fosfaat receptoren zou leiden.

V

Het bestaan van tenminste drie subtypen van somatostatine receptoren in verschillende menselijke weefsels duidt op een gedifferentieerde rol van somatostatine in de fysiologische regulatie van deze weefsels.

VI

Internalisatie van het radioactief gelabelde somatostatine-analoon octreotide is een verklaring voor het feit dat somatostatine receptor positieve tumoren na 48 uur nog zichtbaar zijn op een "in vivo" scan.

VII

Gebrek aan informatie over de genetische constitutie en de metabole "pathways" van de muis kan er toe leiden dat de uitschakeling van een muize gen, via genetische recombinatie (knock out), tegen de verwachting in geen effect heeft.

VIII

Het klassificeren van een gezond individu als patient op grond van zijn of haar genotype is een verkeerde toepassing van DNA diagnostiek.

Beutler and Gelbart (1992) Am. J. Med. Genet. 44: 389

Sidransky et al. (1992) Am. J. Med. Genet. 44: 390

IX

De veranderingen die optreden in een metastaserende tumorcel wat betreft de proteolytische enzymen en celadhesie moleculen bieden aanknopingspunten voor meer gerichte therapie bij uitgezaaide tumoren.

Schultz et al. (1988) Cancer Res. 48: 5539-5545

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DeClerck et al. (1992) Cancer Res. 52: 701-708

X

Architecten bedekken hun fouten met klimop, koks gieten er een extra gekruid sausje over en moleculair biologen gebruiken een phosphor imager.

Vrij vertaald naar een oud Portugees spreekwoord.

XI

Het moet worden voorkomen dat de toekomstige generatie van laboratorium medewerkers kit-kids worden.

XII

WordPerfect 5.1 is not perfect.

