Chapter 1

General introduction and scope of the thesis

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Introduction

The human body contains numerous macromolecular constituents of which many contain sugar moieties. Among these glycoconjugates, glycosphingolipids (GSLs) form a distinct class of molecules. They uniquely consist of a lipid moiety (ceramide) to which a single sugar or a chain of sugar carbohydrates can be linked (Figure 1A). The biosynthesis and versatile functions of GSL have been recently reviewed [1-3], (see also **Chapter 2**).

The simplest GSL is glucosylceramide (GlcCer), ubiquitously present in cells of multi-cellular organisms. It is synthesized by the transfer of glucose from UDP-glucose to ceramide by the enzyme glucosylceramide synthase and acts as precursor of many complex GSLs [4]. GSLs are stepwise degraded in lysosomes of cells. The penultimate step in GSL degradation is deglucosylation of GlcCer to ceramide and glucose. This reaction is catalyzed by the lysosomal acid β-glucosidase, commonly named glucocerebrosidase (GCase; GBA) encoded by the *GBA* gene [5, 6]. Inherited deficiencies in GCase result in lysosomal accumulation of GlcCer, which forms the basis for Gaucher disease (GD) [7], (Figure 1B).

GD is heterogeneous in clinical manifestation, involving symptoms such as hepatosplenomegaly, thrombocytopenia, anemia, skeletal deterioration, osteoporosis, neurological symptoms and aberrant skin barrier properties [7]. Besides the most extreme variant, the collodion baby with lethal skin abnormality, three GD phenotypes are generally discerned: type 1, the non-neuronopathic variant; type 2, the acute (infantile) neuronopathic variant, and type 3, the sub-acute (juvenile) neuronopathic variant (see **Chapter 2** for a review). The complete absence of GCase activity leads to a lethally disrupted skin barrier due the crucial role of GCase in the skin for proper conversion of

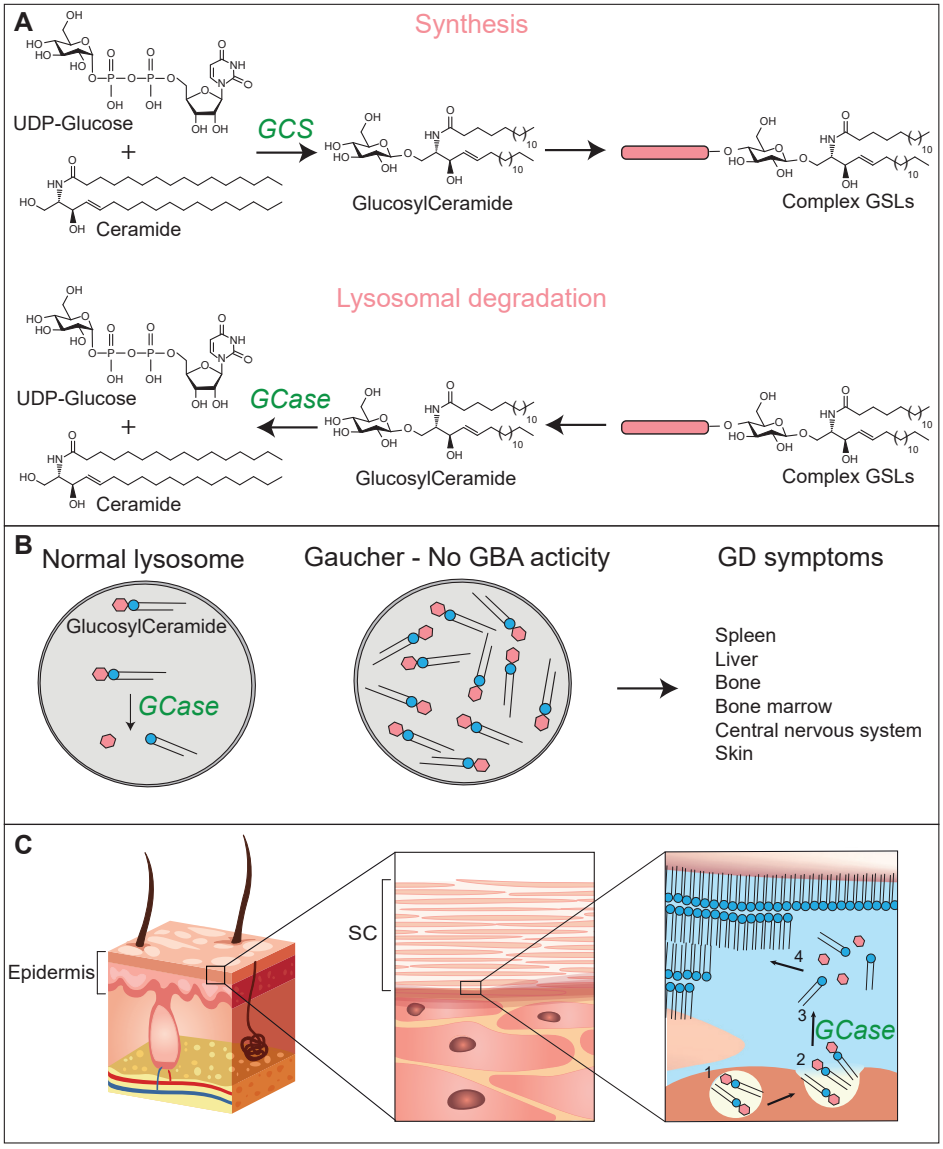

Figure 1: Formation and degradation of glucosylceramide in lysosomes and skin. A. Formation and lysosomal degradation of glucosylceramide (GlcCer). B. Conversion of GlcCer to ceramide by GCase in the lysosome, a lack of GCase activity leads to an accumulation of GlcCer in the lysosome with associated symptoms in GD patients. C. Schematic overview of SC lipid layer formation in the epidermis. 1. Lamellar Bodies (LBs) in the keratinocytes are filled with ceramide precursor GlcCer together with GCase. 2. Extrusion process. 3. Conversion of GlcCer by GCase to ceramides. 4. Formation of the extracellular stacked lipid layers: the lipid barrier in the SC.

skin generating local ceramides [8, 9]. The barrier function of the skin, offering protection against external hazardous factors and preventing water loss, resides in the outer part, the epidermis (Figure 1C).

The outermost part of the epidermis, the stratum corneum (SC), contains a high level GCase [10-12]. The SC consist of corneocytes (cornified keratinocytes) embedded in layers of lipid lamellae in a 'brick-and-mortar" like structure [13]. The lipid lamellae are the 'mortar' and contain approximately 50% ceramides, 25% cholesterol, and 15% free fatty acids with little phospholipid. The GlcCer and sphingomyelin precursors of the SC ceramides are first packed in lamellar bodies (LBs) of keratinocytes along with their hydrolyzing enzymes including GCase. The internal content of LBs (lipids and enzymes) is released by keratinocytes into the SC where ceramides are enzymatically generated (Figure 1C). A proper lipid composition is essential for a correct structure and barrier property of the SC. In various skin diseases, like atopic dermatitis, psoriasis and forms of ichthyosis the SC composition is abnormal [14-16].

Glucocerebrosidase

Following the discovery of the molecular basis of GD in the sixties, GCase has been intensively studied. The features of the enzyme are reviewed in **Chapter 2**. It has become apparent that GCase is a protein of 497 amino acids that contains 4 N-linked glycans (Figure 2A). Contrary to the situation for other lysosomal hydrolases, the glycans of GCase acquire no mannose-6-phosphate recognition signal and consequently the enzyme's transport is normal in Mucolipidoses II and III where mannos-6-phosphate formation is impaired [17]. The delivery of newly formed GCase to lysosomes is mediated by the membrane protein LIMP-2 (Lysosomal integral membrane protein 2) to which folded GCase binds in the endoplasmic reticulum (Figure 2A) [18]. In the acid lysosome, the enzyme is released from its binding partner [19]. Inside the lysosome, GCase is assisted by saposin C in degradation of GlcCer, as illustrated by the Gaucher-like phenotype of individuals with a defective saposin C (Figure 2A) [20].

GCase is a retaining β-glucosidase of which the crystal structure has been resolved (Figure 2B) [21]. In its catalytic TIM-barrel domain, a nucleophile (E340) and acid-base residue (E235) mediate catalysis with a glucosyl-E340 intermediate (Figure 2C) [22]. This reaction-mechanism has allowed the design of activity-based probes (ABPs), existing of cyclophellitol-based compounds that covalently bind to the catalytic nucleophile E340 (Figure 2D) [23]. The mechanism-based labeling of GCase with ABP allows ultrasensitive visualization of active enzyme molecules *in situ* and cross species (see also **Chapter 2**) [24-26].

GCase is encoded by the *GBA* gene (locus 1q21) and numerous mutations have meanwhile been identified in association with GD [7]. Most mutations concern amino acid substitutions of which N370S GCase and L444P GCase are most commonly encountered. The former mutation results in an enzyme with catalytic abnormalities, whereas the latter impairs folding of newly formed GCase in the endoplasmic reticulum which results in markedly reduced lysosomal levels [27].

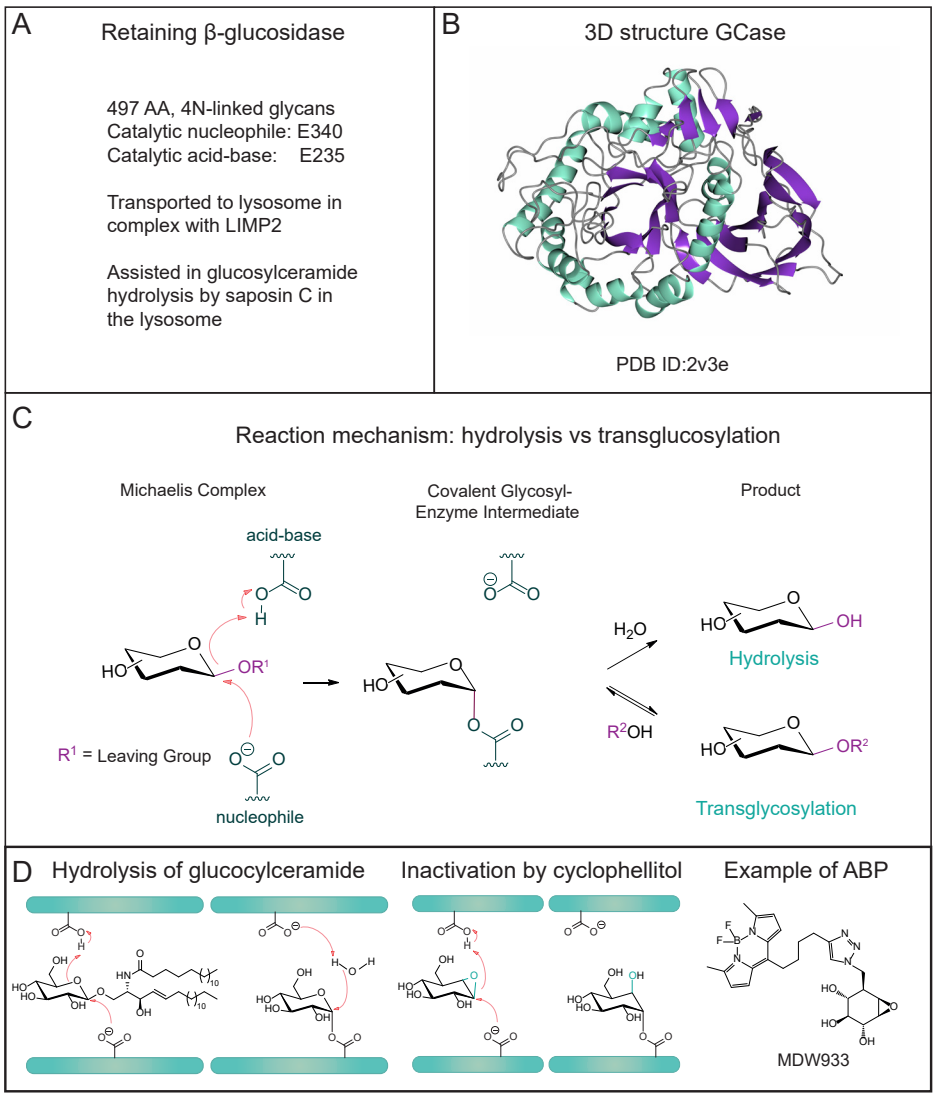

Figure 2: Features of Glucocerebrosidase. A. 3D-structure of GCase. B. Composition, transport and lysosomal activation. C. Reaction mechanisms: hydrolysis versus transglycosylation. D. Mechanism-based suicide inhibition: activity-based probes.

Pathophysiology during GCase deficiency.

Despite the detailed knowledge on GCase, many aspects of the pathophysiology of GD are still poorly understood. For example, while some GCase mutations are associated with more severe disease (L444P GCase) and other with milder and exclusively visceral pathology (N370S GCase), the *GBA* genotype of individual GD patients poorly predicts the precise course of disease and organs involved in this [28]. The nature of the apparent modifiers of disease manifestation is still largely elusive, (see **Chapter 2**).

It has been recognized that adaptive metabolism occurs during GCase deficiency with clinical implications. The characteristic excessive presence of gangliosides in GD patients seems to underly their insulin resistance [29]. Conversion in lysosomes of accumulating GlcCer by acid ceramidase generates glucosylsphingosine (GlcSph) that may leave lysosomes spread through the body of GD patients [30, 31]. Recently, GlcSph is considered as pathogenic, for example in relation to osteoporosis, complement activation and gammopathy that may develop into multiple myeloma [32-34]. The markedly increased risk for Parkinson's disease among carriers of a mutant *GBA* is still unexplained [35]. It has been hypothesized that excessive GlcSph promotes α-synuclein aggregation, a hallmark of Parkinson's disease [36]. However, a clear relation between GlcSph levels and Parkinson's disease does not exist.

Catalytic features of GCase.

In view of the unresolved questions regarding pathophysiology during GCase deficiency, careful investigation of the catalytic features of GCase seems warranted. It has quite recently been recognized that GCase, as reported earlier for other retaining glycosidases, is able to perform tranglucosylation [37, 38]. This reaction implies the transfer of the glucose moiety from the sugar donor (GlcCer) to an acceptor (Figure 2C). It has been demonstrated that cholesterol is a suitable acceptor for GCase. The enzyme may generate glucosylated cholesterol (GlcChol) when cholesterol accumulates in lysosomes, as is the case in Niemann Pick disease type C [38]. At normal conditions, GlcChol is primarily hydrolyzed to cholesterol and glucose by GCase in lysosomes. The physiological relevance of GlcChol is presently unknown.

Another aspect is the potential activity of GCase towards β-xylosides. Many β-glucosidases are known to also acts as β-xylsoidases and this is also the case for GCase. Recent studies (see **Chapter 6**) demonstrate that GCase not only hydrolyzes β-xylosides but also performs transxylosylation and generates xylosylated cholesterol (XylChol). The physiological relevance of recently identified endogenous XylChol and xylosylated ceramide warrants follow-up investigations on the physiological impact of these newly discovered lipids.

Role of GCase beyond the lysosome.

Studies with cultured cells have indicated that normally secretion of GCase molecules is prevented. While other lysosomal hydrolases are partly secreted due to aberrant sorting, this is not apparent for GCase. Only during overexpression some enzyme is secreted, likely due to levels exceeding the capacity of the binding partner LIMP-2. One important exception in this respect form keratinocytes. As discussed above, in these cells GCase is partly sorted to LBs to be actively delivered into the acid extracellular space of the SC where it generates essential ceramide molecules from secreted GlcCer precursor lipids (see **Chapter 3**). Other roles for the high amounts of extracellular GCase in the SC deserve attention (**Chapter 5**).

Visualization of active GCase.

A multitude of techniques has become available over the years to visualize active GCase molecules. Activity assays employing GlcCer that is radioactively labeled in the glucose or ceramide moiety to monitor enzyme levels in biological materials are available for some decades [5]. Great use has also been made of artificial GlcCer with a fluorescent acyl-NBD moiety allowing visualization of active enzyme when combined with chromatography methods (high performance liquid chromatography (HPLC) and high-performance thin layer chromatography (HPTLC)) [39]. Likewise, isotope-encoded GlcCer and ultra-performance liquid chromatography – tandem mass spectrometry (ULC-MS/MS) may be employed. Feeding of cells with radioactive serine and with isotope encoded or radioactive sphinganine or sphingosine when used in combination with available specific inhibitors of the GCase allow the analysis of GCase activity *in vivo*. Given their convenient use, popular artificial substrates for GCase activity assays are chromogenic para-nitrophenyl-β-glucoside and fluorogenic 4-methylumbelliferyl-β-glucoside [40].

Of particular interest is the visualization of active GCase *in situ*. Antibodies directed against GCase have been widely employed to visualize GCase molecules with histochemistry, immunofluorescence, and immune-electron microscopy [23, 41]. However, these methods do not discern inactive and active enzyme molecules. The recently availability of highly specific ABP directed towards GCase has opened up new possibilities to study the presence of active GCase molecules *in situ*. Examples is the recent application of ABPs to visualize endogenous GCase, and endocytosed exogenous therapeutic enzyme in lysosomes with correlative light and electron microscopy [42]. Along the same line is the visualization with ABP of active GCase in the SC of skin [43], (**Chapter 3**).

For the detection of activity of GCase towards β-xylosides specific assays have been developed, see **Chapter 6**. The commercial fluorogenic substrate 4-mehylumbelliferyl-β-xyloside can be used to monitor β-xylosidase activity exerted by GCase, in particular in combination with specific inhibitors of the enzymes to account for contaminating activity of other enzymes in samples.

Specific assays have been developed to monitor transglycosylation activity of GCase (Figure 3), see **Chapter 6**. One method is HPTLC based separation of glycosylated product of NBD-cholesterol that is quantified with a Typhoon (Fluorescence mode, Emission 520 BP, laser 488 nm). Alternatively, LC-MS/MS can be employed to detect sensitively and specifically formed trans-products. With this method liquid chromatography is used to separate metabolites of interest. The effluent from the column is ionized in charged particles that migrate under vacuum through the quadrupoles (mass analyzers). A preset mass/charge (m/z) is targeted to pass through the first quadrupole: the parent (precursor) ion. All other particles with a different m/z ratio are then excluded. The selected ions are then fragmented in the collision cell: the daughter ions

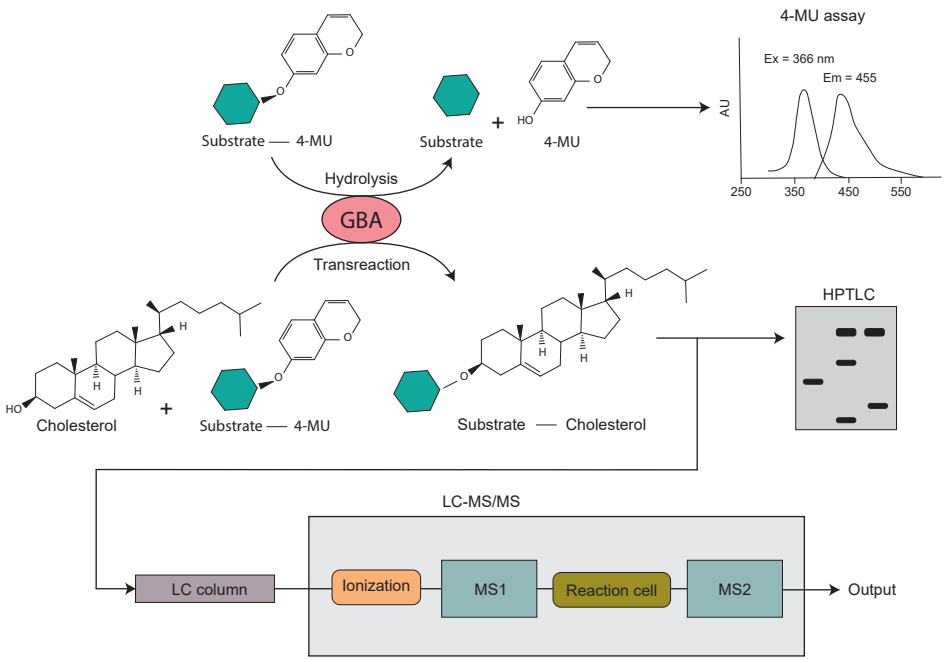

Figure 3: Method to determine in parallel hydrolysis and transglucosylation by GCase. Hydrolysis can be determined by measuring the released fluorescent 4-methylumbelliferone (4-MU) from the non-fluorescent substrate. Transglucosylation by GCase can be in parallel visualized by separation of transproduct using high-performance thin-layer chromatography (HPTLC) or mass spectrometry (MS).

(product ions). The final quadrupole isolates specific daughters followed by quantification via an electron multiplier (Figure 3). This selection of a parent ion with a particular m/z at the first stage and (multiple) daughter ions in the final stage is also referred to as Multiple Reaction Monitoring (MRM, or Selective Reaction Monitoring; SRM).

Analysis of glycolipids.

LC-MS/MS is a powerful method to identify and quantify complex lipids in biological materials. The availability of isotope-encoded standards of lipids of interest is of great value in this respect [44, 45]. Analysis of the lipid composition of the SC of the skin is particularly challenging. The SC can be isolated by tape stripping of the skin or by dermatoming skin and subsequent isolation of the SC by tryptic digestion. Alternatively, skin biopsies from subjects followed by tryptic digestion can be used to isolate the SC. Lipids can be subsequently extracted from SC, as from other tissues.

Challenges.

At present costly therapeutic interventions are available to treat visceral symptoms of GD such as hepatosplenomegaly and hematological symptoms. These therapies are based on supplementation of macrophages with intravenously infused recombinant GCase (enzyme replacement therapy; ERT) or the oral administration of small compound inhibitor of GlcCer synthesis (substrate reduction therapy; SRT) [46-48]. In clinical trials are compounds aiming to stabilize and promote folding of mutant GCase in amenable GD patients (enzyme enhancement therapy, pharmacological chaperone therapy) [49, 50], see **Chapter 2**. Present therapies fail to prevent bone disease and central nervous system manifestations during GCase deficiency. A better understanding of the function of GCase at various body locations and detailed knowledge on its substrates and products is required to improve treatment of GCase deficiency in the future.

Scope of the thesis investigations

The central aim of the undertaken studies was to increase insight in the catalytic versatility and potential functions of glucocerebrosidase (GCase), inside and beyond the lysosome.

Chapter 2 provides a review of the present knowledge on GCase, covering its composition, life cycle and roles in the lysosome and beyond the organelle in the skin. Attention is also paid to the metabolism of glycosphingolipids and Gaucher disease.

Chapter 3 describes the application of ABPs to visualize active GCase *in situ* in skin. The sensitivity of the newly developed method is compared to zymography. The investigation confirmed the presence of high amounts of active GCase at the interface of the vital epidermis and SC. In addition, the effect of inhibition of GCase on GlcCer and ceramide in a 3D-cultured skin model was investigated.

Chapter 4 reports the visualization of active GCase in SC samples of skin from normal individuals and those suffering from atopic dermatitis. In parallel to ABP-labelled GCase, acid sphingomyelinase was visualized by zymography. The data on localization of the ceramide-generating enzymes were compared with measured lipid composition of SC samples. The study illustrated the relation between correct localization of (active) enzymes and desired SC lipid composition for proper skin barrier function.

Chapter 5 deals with the occurrence of GlcChol in the skin, the glucosylated sterol that can be formed by GCase via transglucosylation using GlcCer as glucose donor and cholesterol as acceptor. The investigation revealed the presence of GlcChol in SC. This finding is not surprising given the local abundance of GCase, GlcCer and cholesterol.

Chapter 6 presents an investigation on the ability of GCase to metabolize β-xylosides. It is demonstrated that GCase can hydrolyze various β-xylosides. Moreover, it can use β-xylosides as sugar donor in the transglycosylation of cholesterol, rendering XylChol. The natural presence of XylChol in cells and tissues was subsequently demonstrated. In search for a natural β-xyloside metabolite that acts as sugar donor in the formation of XylChol, a completely novel glycosphingolpid was identified: xylosylated ceramide (XylCer). XylCer was found to be generated by glucosylceramide synthase using UDP-xyloside as sugar donor.

Chapter 7 discusses the findings of the conducted investigations in view of the literature. Moreover, it proposes future investigations on GCase and other β-glucosidases, in and beyond the lysosome.

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