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## Contents

Introduction .....	121
Databanks .....	122
Name and History .....	122
Structure .....	123
Enzyme Activity Assay and Substrate Specificity .....	123
Preparation .....	124
Biological Aspects .....	124
Knockout Mouse and Transgenic Mice .....	125
Human Disease .....	126
Future Perspectives .....	126
Cross-References .....	127
Further Reading .....	127
References .....	127

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## Introduction

Isoglobotriaosylceramide or isogloboside 3 (iGb3) has been the subject of intense research by numerous laboratories and has provoked lively debate in the literature since it was suggested to be the endogenous ligand involved in thymic selection of a subset of natural killer T cells (iNKT) in both mice and humans. iGb3 is the first member of the isoglobo-series glycosphingolipids and is synthesized by alpha 1,3-galactosyltransferase 2.

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## Databanks

IUBMB enzyme nomenclature: EC 2.4.1.87

Alpha 1,3-galactosyltransferase 2, pseudogene (A3GALT2P)

Species	Gene symbol	GenBank accession number	UniProt ID	PDB accession number
<i>Mus musculus</i>	A3galt2	NC_000070.6	Q3V1N9	N/A
<i>Rattus norvegicus</i>	A3galt2	NC_005104.3	A0A4Z3	N/A
<i>Homo sapiens</i>	A3GALT2P	NG_012483	N/A	N/A

## Name and History

Alpha 1,3-galactosyltransferase 2, also referred to as isoglobotriaosylceramide synthase or iGb3 synthase (iGb3S), catalyses the transfer of galactose (Gal) to lactosylceramide (Lac-Cer) to form iGb3 (Gal $\alpha$ 1,3Gal $\beta$ 1,4Glc-Cer). Here we will refer to this galactosyltransferase as iGb3S.

The glycosphingolipid iGb3 was originally chemically identified in rat spleen (Stoffyn et al. 1973b). Subsequently iGb3 has been found in dog intestine (Sung and Sweeley 1979), non-epithelial cells of the small intestine of the rat (Angstrom et al. 1982b; Breimer et al. 1982), in the small intestine of the cat (Teneberg et al. 2004), and more recently in the dorsal root ganglion (Speak et al. 2007), thymus (Milland et al. 2006; Li et al. 2009; Porubsky et al. 2012), and dendritic cells (Li et al. 2009) of the mouse. Furthermore, a fucosylated form of iGb3, Fuc $\alpha$ 1,2Gal $\alpha$ 1,3Gal<sup>®</sup>1,4Glc-Cer, has been identified in the pig (Slomiany et al. 1974; Diswall et al. 2007; Puga Yung et al. 2012) and the rat large intestine (Hansson et al. 1980). In addition, novel branched or hybrid-type variants of the isogloboside series have also been identified in rat (Hansson et al. 1987), horse (Yamamoto et al. 1999), and salmon (Niimura 2006).

Other glycosyltransferases can utilize iGb3 as a substrate to produce other glycosphingolipids. The next member of the isogloboside series, iGb4 GalNAc $\alpha$ 1,3Gal $\alpha$ 1,3Gal $\beta$ 1,4Glc-Cer also known as cytolipin R, has been identified in the small intestine of the rat (Angstrom et al. 1982a; Breimer et al. 1982), rat lymphosarcoma (Laine et al. 1972), and in the rat kidney (Siddiqui et al. 1972) as well as in the thymus and dendritic cells of the mouse (Li et al. 2009).

The cDNA clone encoding iGb3S was originally isolated from a rat placental cDNA library by expression cloning using Chinese hamster ovary (CHO) cells (Keusch et al. 2000). Other members of the isogloboside series, iGb4 and iGb5, are produced in transfected CHO cells (Keusch et al. 2000). Mouse iGb3S has also been isolated from GGTA1<sup>-/-</sup> thymus mRNA (Milland et al. 2006).

## Structure

The amino acid sequence of iGb3S assigns it to the Family 6 of glycosyltransferases [as defined by the Glycosyltransferase Family Server of the CAZy (carbohydrate-active enzymes) database] that are all retaining enzymes that transfer either  $\alpha$ Gal or  $\alpha$ GalNAc. Other members of this family include the  $\alpha$ 1,3-galactosyltransferase 1 (GGTA1), the A and B blood group glycosyltransferases, and the Forssman synthase (Milland and Sandrin 2006). iGb3S is a type II integral membrane protein of 339 amino acids in the rat (Keusch et al. 2000) and 370 amino acids in the mouse (Milland et al. 2006). The difference in size is due to the cytoplasmic domain of the mouse being atypically long for a glycosyltransferase, having an insertion of 31 amino acids before the conserved  $^{38}$ RAKKR $^{42}$  flanking the cytoplasmic domain (Milland et al. 2006). The transmembrane domain of the rat is one residue larger (19 amino acids) than the mouse. The C-terminal catalytic domain, containing the essential DVD sequence, has 91 % amino acid identity between these species, with a conserved pattern of cysteine residues (Keusch et al. 2000; Milland et al. 2006). In both rat and mouse, the iGb3S coding regions are encoded by five exons with conserved exon and intron lengths and consensus sequences at the exon-intron junctions (Milland et al. 2006).

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## Enzyme Activity Assay and Substrate Specificity

An in vitro assay for this enzyme was developed to examine substrate specificity using transfected cell lysates and supernatants (Keusch et al. 2000). The reaction buffer was 100 mM sodium cacodylate pH 6.8, 0.5 % Triton X-110, 5 mM ATP, 250  $\mu$ M UDP-Gal, UDP- $^{3}$ H]Gal, 15 mM MnCl<sub>2</sub>, and protease inhibitors in a final volume of 100  $\mu$ l. Cell lysates/supernatants and acceptor substrates were incubated at 37 °C for 90 min to overnight, before reverse-phase chromatography using Sep-Pak C18 cartridges and analysis by TLC. Using this assay, iGb3S was able to utilize Lac-Cer, Gal-Cer, and Gb3 to produce Gal $\alpha$ 1,3Gal $\beta$ 1,4Glc-Cer (iGb3), Gal $\alpha$ 1,3Gal-Cer, and Gal $\alpha$ 1,3Gal $\alpha$ 1,4Gal $\beta$ 1,4Glc-Cer, respectively (Keusch et al. 2000) (See Fig. 12.1).

Transfection of the rat iGb3S into CHOP cells (Chinese hamster ovary cells transformed with polyoma large T antigen) results in the synthesis of poly- $\alpha$ 1,3Gal glycolipids (Taylor et al. 2003). Two types of poly- $\alpha$ 1,3Gal glycosphingolipids have been identified chemically in the rat (Angstrom et al. 1982a, b; Breimer et al. 1982; Ariga et al. 1989) and cat (Teneberg et al. 2004), found either on Gb3 or iGb3 (Fig. 12.1), and it is highly likely that these are produced by iGb3S. For Gb3 the number of  $\alpha$ 1,3Gal moieties identified ranges from 1 to 5 (Angstrom et al. 1982a, b; Breimer et al. 1982; Ariga et al. 1989), and for iGb3, one  $\alpha$ 1,3Gal has been identified (Teneberg et al. 2004), although the data from Taylor suggest up to five additional  $\alpha$ 1,3Gal can be added to iGb3 (Taylor et al. 2003). In addition,

Substrate	Product
1. Lac-Cer	Gal $\alpha$ 1,3Gal $\beta$ 1,4Glc-Cer
2. Gal-Cer	Gal $\alpha$ 1,3Gal-Cer
3. Gb3	Gal $\alpha$ 1,3Gal $\alpha$ 1,4Gal $\beta$ 1,4Glc-Cer
	Poly $\alpha$ 1,3Gal variants
	Gal $\alpha$ 1,3Gal $\alpha$ 1,3Gal $\alpha$ 1,4Gal $\beta$ 1,4Glc-Cer
	Gal $\alpha$ 1,3Gal $\alpha$ 1,3Gal $\alpha$ 1,3Gal $\alpha$ 1,4Gal $\beta$ 1,4Glc-Cer
	Gal $\alpha$ 1,3Gal $\alpha$ 1,3Gal $\alpha$ 1,3Gal $\alpha$ 1,3Gal $\alpha$ 1,4Gal $\beta$ 1,4Glc-Cer
	Gal $\alpha$ 1,3Gal $\alpha$ 1,3Gal $\alpha$ 1,3Gal $\alpha$ 1,3Gal $\alpha$ 1,3Gal $\alpha$ 1,4Gal $\beta$ 1,4Glc-Cer
4. iGb3	Gal $\alpha$ 1,3Gal $\alpha$ 1,3Gal $\beta$ 1,4Glc-Cer

**Fig. 12.1** The different substrates utilized by iGb3S. Substrates shown on *left* and products on the *right*. See text for references

globoside-like terminated forms (terminating in GalNAc $\beta$ 1,3) of the poly- $\alpha$ 1,3Gal glycolipids have also been identified (Angstrom et al. 1982a; Breimer et al. 1982) (Fig. 12.2).

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## Preparation

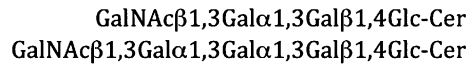
Unlike other glycosyltransferases, protocols to purify iGb3S from tissues have not been developed. However, iGb3 has been synthesized *in vitro* using lactosylceramide and microsomes isolated from rat spleen cells (Stoffyn et al. 1973a), rat bone marrow cells (Stoffyn et al. 1973a), and rat kidney cells (Stoffyn et al. 1974). More recently, a recombinant form of mouse iGb3S has been produced in insect cells and used to synthesize iGb3 *in vitro* (Zhou et al. 2004).

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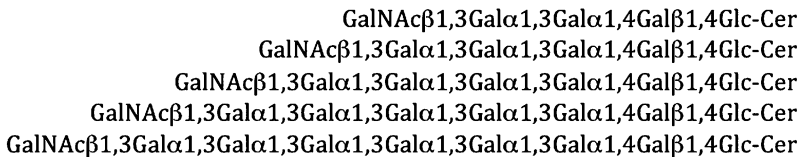
## Biological Aspects

Interest in a potential biological role for iGb3 arose from the observation that a glycosphingolipid isolated from the marine sponge *Agelas mauritanus*,

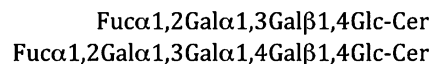
### 1. GalNAc of isoglobosides



### 2. GalNAc of poly- $\alpha$ -galactosylated Gb3



### 3. Fucosylated isoglobosides



**Fig. 12.2** Additional modifications of iGb3S products

$\alpha$ -galactosylceramide ( $\alpha$ -Gal-Cer), was a potent agonist for NKT cells in a CD1d-dependent manner in both mice and humans (Kawano et al. 1997; Brossay et al. 1998). However, as this glycosphingolipid is not produced in mammals, the physiological relevance was unclear. Subsequently, several glycosphingolipids with terminal  $\alpha$ Gal were examined for their ability to activate iNKT cells. This resulted in iGb3 being proposed to be the main endogenous ligand responsible for iNKT cell development and self-recognition in both mice and humans (Zhou et al. 2004). However, this was initially challenged by two publications (Porubsky et al. 2007; Speak et al. 2007). Using a highly sensitive HPLC assay, Speak et al. failed to detect iGb3 in mouse or human thymus (Speak et al. 2007). Whereas Porubsky et al. more strongly challenged the significance of iGb3 in mouse iNKT cell development by reporting normal numbers of NKT cells in the thymus of iGb3<sup>-/-</sup> mice (Porubsky et al. 2007).

Membrane bound glycolipids are known to be attachment sites for bacteria and bacterial toxins (Karlsson 1989), and products of iGb3S are no exceptions: enterohemorrhagic *Escherichia coli* have been shown to bind to Gal $\alpha$ 1,3Gal $\alpha$ 1,3Gal $\beta$ 1,4Glc-Cer, but not Gal $\alpha$ 1,3Gal $\alpha$ 1,4Gal $\beta$ 1,4Glc-Cer isolated from cat small intestine (Teneberg et al. 2004).

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## Knockout Mouse and Transgenic Mice

iGb3 synthase knockout (iGb3S<sup>-/-</sup>) mice have been generated by targeting the coding sequence of exon 5 and replacing this with a neomycin selection cassette in C57BL/6 ES cells (Porubsky et al. 2007). Homozygous iGb3S<sup>-/-</sup> mice grow and

breed normally, with no evident signs of developmental or behavioral defects (Porubsky et al. 2007). Furthermore, these mice had normal iNKT cells numbers (in the thymus, spleen, and liver) and TCR Vb usage. In addition, iNKT cells and dendritic cells from either iGb3S<sup>-/-</sup> or wild-type mice responded to  $\alpha$ -Galcerin in an identical manner (Porubsky et al. 2007). These data strongly suggest that iGb3 is not the endogenous ligand for iNKT cell selection.

To date there are no reports regarding iGb3S transgenic mice.

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## Human Disease

The issue of iGb3 expression in humans is of major importance to both xenotransplantation and NKT cell biology. If humans express iGb3S, iGb3 lipid on transplanted pig tissues would not pose a problem, as tissue would not be recognized as foreign. Conversely, if humans do not express functional iGb3S, then expression of iGb3 on pig cells could lead to NKT cell activation resulting in destruction of the xenograft. Although it has been suggested that human thymus express extremely low levels of iGb4 (and therefore by inference iGb3) (Li et al. 2008), extensive analysis of several human tissues (heart, lung, kidney, spleen, and thymus) failed to detect spliced iGb3S mRNA (Christiansen et al. 2008). Indeed, expression of chimaeric molecules, containing the catalytic domain of human iGb3S with the remaining portion from rat iGb3S (cytoplasmic tail to stalk region), were unable to synthesize iGb3 (Christiansen et al. 2008). Furthermore, site-directed mutagenesis used to analyze which amino acid(s) contributed to the loss of function showed that substitution of rat Y<sup>252</sup>N resulted in the complete elimination of iGb3, whereas L<sup>187</sup>P showed a significant reduction (typically 70–95 %). Reverse mutation of the nonfunctional chimaeric human iGb3S to their rat equivalents with either point mutation alone (i.e., P<sup>187</sup>L or N<sup>252</sup>Y), or in combination (P<sup>187</sup>L+N<sup>252</sup>Y), did not lead to a gain of function, implying that human iGb3S must have other mutations that are important for its inactivation (Christiansen et al. 2008). Thus, even if human iGb3S was expressed at either the mRNA or protein level, it would be nonfunctional due to several mutations that differentiate the human enzyme from its functional counterpart in the rat, and therefore, human *A3GALT2P* is a non-processed pseudogene.

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## Future Perspectives

The potential role of iGb3 as the principle endogenous ligand for iNKT cell development and function in mice and, albeit indirectly, in humans still represents one of the most important and controversial issues in the iNKT cell field. A fundamental question that remains to be answered is if iGb3 is expressed in humans due to an unidentified functional allele, does this have any significance in iNKT biology? Another intriguing question is the molecular basis for the unique characteristic of iGb3S to utilize multiple substrates with different anomeric configurations.

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## Cross-References

- ▶ [Glycoprotein Alpha 1,3-Galactosyltransferase 1, Pseudogene \(GGTA1P\)](#)
- ▶ [Histo-blood Group A and B Transferases, Their Gene Structures, and Common O Group Gene Structures](#)
- ▶ [UDP-Gal: Ceramide Galactosyltransferase \(UGT8\)](#)

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## Further Reading

- Christiansen et al. 2008: First publication to show that humans lack iGb3 due to the absence of functional iGb3S.
- Keusch et al. 2000: First report on cloning of iGb3S.
- Milland et al. 2006: Demonstrated the molecular basis for Gal $\alpha$ (1,3)Gal expression in animals with a deletion of the GGTA1 gene.
- Porubsky et al. 2007: Demonstrated normal iNKT cell development in iGb3S knockout mice.
- Speak et al. 2007: Described lack of iGb3 in human thymus.
- Zhou et al. 2004: Suggested that iGb3 was the main endogenous ligand responsible for iNKT cell development and self-recognition in both mice and humans.

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