Glucosylceramide synthase (GlcT-1) catalyzes the formation of glucosylceramide (GlcCer), the core structure of major glycosphingolipids (GSLs), from ceramide and UDP-glucose. Ceramide and its metabolites, such as sphingosine-1-phosphate, are now known to be important mediators of apoptosis and cell survival. Recently, we have shown that GlcT-1 functions to regulate intracellular ceramide levels via glycosylation of ceramide. In this study, we employ the fruit fly Drosophila melanogaster as a model system for understanding the in vivo roles of GlcT-1. We isolated and characterized a GlcT-1 homologue (DGlcT-1) from Drosophila. When DGlcT-1 was expressed in GM-95 cells deficient in GSLs (because of the absence of GlcT-1 activity), these cells regained the ability to synthesize GSLs. Northern blot and in situ hybridization analyses revealed that the expression of DGlcT-1 mRNA was ubiquitous throughout development, suggesting that DGlcT-1 is important for development and differentiation. Indeed, RNA interference experiments demonstrated that the loss of GlcT-1 function enhances apoptotic cell death. Conversely, targeted expression of GlcT-1 partially rescued cell death caused by the proapoptotic factors Reaper and Grim, suggesting that ceramide generation might be one signal pathway that executes the cell death program. We also found that GlcT-1 localized not only in the Golgi apparatus but also in the perinuclear endoplasmic reticulum, providing the first visual evidence of GlcT-1 in membranes. These results indicate that GlcT-1 might down-regulate ceramide generated in these membranes.

Ceramide and its metabolites, such as sphingosine-1-phosphate, have emerged as novel second messengers for intracellular signaling pathways that are involved in regulation of diverse cellular responses to exogenous stimuli (1–3). Its mechanisms of action and the regulation of its production have focused particular attention since ceramide has been shown to be an intracellular effector in apoptosis (4, 5). However, the role of ceramide in apoptosis has been the subject of some controversy. Whether ceramide generation precedes induction of apoptosis or is subsequent to the commitment process is uncertain (6). Very recently, Acharya et al. (7) have shown that retinal degeneration caused by light-dependent photoreceptor cell death involves ceramide generation. It remains unclear, however, where ceramide is formed in the visual system. Moreover, what ceramide targets and its mode of action also are unknown. Importantly, cell death in this system is completely rescued by the introduction of ceramidase, an enzyme that hydrolyzes ceramide to sphingosine and fatty acid.

Glucosylceramide synthase (GlcT-1) EC 2.4.1.80 catalyzes the transfer of glucose (Glc) from UDP-Glc to ceramide (Cer) to form glucosylceramide (GlcCer). We propose that GlcT-1 has dual functions. First, GlcT-1 can act as a negative regulator of ceramide-mediated reactions by modifying ceramide with glucose. GlcT-1-mediated glycosylation of ceramide reduces intracellular ceramide level and protects cells from apoptosis (8–11). Second, GlcT-1 can catalyze the synthesis of GlcCer, the core structure of major glycosphingolipids (GSLs). GSLs form lipid microdomains (e.g. rafts of the plasma membrane) that have been implicated in various important cellular processes, such as differentiation, adhesion, proliferation, and cell-cell recognition (12–14). Thus, GlcT-1 plays significant roles in a variety of biological processes by regulating both intracellular ceramide levels and the overall synthesis of GSLs.

We have previously cloned cDNAs of human GlcT-1 (HGlcT-1, UGGC) (15) and mouse GlcT-1 (MGlcT-1) (16). GlcT-1 is a type III membrane protein. Its catalytic domain is located at the cytosolic surface of the Golgi apparatus (17–20); thus, GlcT-1-mediated reactions occur on the cytosolic surface. Other glycosylation reactions involved in GSL synthesis, however, take place on the luminal surface of the Golgi (21, 22). Under in vitro conditions, cells apparently do not require the de novo synthesis of GlcCer for cellular proliferation and survival.

Received for publication, January 15, 2004, and in revised form, May 24, 2004

Published, JBC Papers in Press, June 21, 2004, DOI 10.1074/jbc.M400444200

This paper is available on line at http://www.jbc.org

Drosophila Glucosylceramide Synthase

A NEGATIVE REGULATOR OF CELL DEATH MEDIATED BY PROAPOPTOTIC FACTORS*

Ayako Kohyama-Koganeya*, Takeshi Sasamura*, Eriko Oshima*, Emiko Suzuki*, Shoko Nishihara**, Ryu Ueda**,††, and Yoshio Hirabayashi***

From the ‡Neuronal Circuit Mechanisms Research Group, Brain Science Institute, The Institute of Physical and Chemical Research (RIKEN), Wako-shi, Saitama 351-0198, Japan, the §Department of Biological Science and Technology, Tokyo University of Science and the ¶Department of Fine Morphology, The Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo 108, Japan, the ¶Laboratory of Cell Biology, Department of Bioinformatics, Faculty of Engineering, Soka University, Hachioji, Tokyo, 192-8577, Japan, the §§Invertebrate Genetics Laboratory, National Institute of Genetics, Mishima, Shizuoka 441-8540, Japan, and the ***Core Research for Evolutional Science and Technology (CREST) of Japan Science and Technology Corporation (JST), Kawaguchi, Saitama 332-0012, Japan

The abbreviations used are: GlcT-1, ceramide glucosyltransferase; GSL, glycosphingolipid; GlcCer, glucosylceramide; GM3, sialyllactosylceramide; C5′-NBD-Cer, 6-[(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino] caproyl}sphingosine; SM, sphingomyelin; SMase, sphingomyelinase; GMR, glass multimer promoter; ER, endoplasmic reticulum; RACE, rapid amplification of cDNA ends; ds, double stranded; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling; TM, transmembrane.

* This work was supported by Grant-in-aid 12140201 (Scientific Research on Priority Areas (B) (to Y. H.) from the Ministry of Education, Culture, Sports, Science and Technology) and the Brain Science Institute, RIKEN, Wako-shi, Saitama 2-1 Higashiwara, Wako-shi, Saitama 351-0198, Japan, Tel./Fax: 81-48-467-6372; E-mail: hirabay@postman.riken.jp.

** To whom correspondence should be addressed: Neuronal Circuit Mechanisms Research Group, Brain Science Institute, The Institute of Physical and Chemical Research (RIKEN), 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan. Tel./Fax: 81-48-467-6372; E-mail: hirabay@postman.riken.jp.

†† The abbreviations used are: GlcT-1, ceramide glucosyltransferase; GSL, glycosphingolipid; GlcCer, glucosylceramide; GM3, sialyllactosylceramide; C5′-NBD-Cer, 6-[(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino] caproyl}sphingosine; SM, sphingomyelin; SMase, sphingomyelinase; GMR, glass multimer promoter; ER, endoplasmic reticulum; RACE, rapid amplification of cDNA ends; ds, double stranded; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling; TM, transmembrane.
The mouse melanoma cell line GM-95 inherently lacks GlcT-1 activity and grows without GSLs (23). However, when GlcT-1 function is knocked out in mice via gene-targeting techniques, these mice die at embryonic day 8 (24). Moreover, enhanced apoptosis is evident in the ectodermal layer of these mice. These observations clearly point to an essential role of GSL in apoptosis. In the ectodermal layer of these mice, it is difficult to explain why the elimination of the GlcT-1 gene is lethal during embryonic development in mice.

Since molecular, genetic, and cell biological techniques are fully available for Drosophila, it is a very attractive alternative to vertebrate systems for examining the functions of Glc-T-1. Despite having a different ceramide composition in the hydrophobic portions, membranes in Drosophila contain sphingolipids similar to those found in mammalian membranes (25). In addition, many components of cell death pathways in mammals are also conserved in Drosophila.

To identify the in vivo roles of Glc-T-1 through genetic manipulation, we first cloned and characterized a homologue of MgGlcT-1 from Drosophila melanogaster, termed DGlCt-1. Then, we examined whether the ectopic expression or elimination of DGlCt-1 affects Drosophila development. Our data suggest that Glc-T-1 functions as a regulator of intracellular ceramide levels, which in turn regulates cell death during Drosophila development.

**EXPERIMENTAL PROCEDURES**

**Cloning of DGlCt-1**—A putative DGlCt-1 genomic DNA sequence was found in P1 clone DS00642 (accession no. AC004365) by searching nucleotide and protein databases (National Center for Biotechnology Information). A CDNA library of the adult Drosophila genome was prepared from Marathon-Ready™ cDNA (Clontech) and adult Drosophila poly(A) RNA (Clontech). The full sequence of DGlCt-1 cDNA was determined from three RACE products derived from the Marathon-Ready/adult Drosophila cDNA library.

**Plasmids**—The coding sequence of DGlCt-1 was amplified by reverse transcription-polymerase chain reaction (RT-PCR) of adult Drosophila poly(A) RNA (Clontech). The cDNA encoding full-length DGlCt-1 was cloned into one of three expression vectors (pcDNA3.1, pcDNA3.1/V5-His-TOPO (Invitrogen), or pUAST) (26). HGlCt-1 deletion mutants were generated with PCR and cloned into either pcDNA3.1/V5-His-TOPO (Invitrogen), or pUAST) (26). HGlCt-1 independent lines were obtained. The following transgenic fly strains were used for phenotypic analysis and genetic interactions: GMR-GAL4, GMR-Grim, GMR-rpr, and GMR-hid (kind gifts from M. Okabe, T. Adachi-Yamada, and Y. Nishida). All crosses were performed at 25 °C.

**RESULTS**

**Identification and Characterization of DGlCt-1**—Through an exhaustive TBLASTN search of the Drosophila sequence data base, we identified a sequence that was similar to the sequence of the GlcT-1 family of genes in the genomic region 58A4–58B1. This region was represented in P1 clone DS00642, which was sequenced in full. cDNA encoded by the Drosophila homologue (DGlcT-1) of GlcT-1 was obtained by RACE. Analysis of its nucleotide sequence revealed an open reading frame of 440 amino acids with a predicted relative molecular mass of 49 kDa. Comparison of cDNA and genomic sequences revealed that this gene did not possess an intron. To date, GlcT-1 from animals, fish, fungi, plants, and bacteria (30) have either been cloned or identified (e.g., humans, Ref. 15; mice, Ref. 16; rats, Ref. 31; Caenorhabditis elegans, Ref. 21; the fungi P. pastoris and M. grisea, Ref. 30; and the plant G. arboreum, Ref. 30) (Fig. 1). The sequence identities of human, mouse, rat, C. elegans, fungi, and plant GlcT-1 are 47, 46, 46, 34, 26, and 21%, respectively. As indicated in Fig. 1, DGlcT-1 possesses motifs essential for catalytic activity such as D1, D2, and D3 and also hypothetical “nucleotide recognition domains” NRD2L and NRD2S (30, 31).

DGlcT-1 Can Catalyze the Formation of GlcCer—To determine whether DGlcT-1 is enzymatically capable of generating GlcCer, lysates from GM-95 cells stably expressing DGlcT-1 (i.e. DGlcT-1 cells) were assayed for GlcCer synthetic activity using C6-NBD-Cer as a substrate (Fig. 2). We also assayed lysates from the parental cell line from which GM-95 cells are derived, MEB4 cells; these have endogenous UDP-Glc. As expected, MEB4 cells (lanes 1 and 2) synthesized C6-NBD-GlcCer, even in the absence of UDP-Glc (lane 1). Neither GM-95 cells (lane 3) nor themock transfectant of GM-95 cells (lane 4)
synthesized C6-NBD-GlcCer. However, DGlcT-1 cells (lane 5) did synthesize C6-NBD-GlcCer. Since the normal body temperature of Drosophila is 25 °C, we also examined the enzymatic activity of DGlcT-1 at 25 °C (lane 7) (Fig. 2). There was no significant difference in the synthetic activity of DGlcT-1 at 25 and 32 °C (lanes 5 and 7).

Although GM-95 cells are deficient in GlcT-1 activity, other enzymes involved in GSL synthesis remain intact. In these cells, these complementary enzymes are capable of restoring the production of GM3, the end product of GSL synthesis. Thus, we analyzed the expression of GM3 in DGlcT-1 cells using flow cytometry and the anti-GM3 mAb M2590 (Fig. 3). GM3 was as strongly expressed in DGlcT-1 cells as in MEB4 cells. This restoration of GM3 expression was not detected in the mock transfectants. GM3 was also detected in DGlcT-1 cells using TLC immunostaining with M2590 (data not shown). These results further demonstrate that DGlcT-1 is capable of catalyzing the formation of GlcCer.

DGlcT-1 Is Expressed Ubiquitously—The expression of DGlcT-1 mRNA was analyzed by Northern blot analysis (Fig. 4A). A single 2.6-kb transcript, consistent with the size of DGlcT-1 cDNA, was detected at embryonic and adult stages of development. The expression pattern of DGlcT-1 mRNA during Drosophila embryogenesis was examined via in situ hybridization of whole mount embryos. DGlcT-1 mRNA was expressed ubiquitously during all embryonic stages (Fig. 4B). With the exception of the central region of the embryo, which mainly contains yolk, DGlcT-1 mRNA was found in essentially all cells during germ band extension (midstage). GlcT-1 is also ubiquitously expressed throughout embryonic development in mammals (15). The expression of DGlcT-1 during all stages of embryonic development in Drosophila suggests that DGlcT-1 may be essential for embryogenesis.

DGlcT-1 Localizes in Both Golgi and ER Membranes—Ceramide can be found in various membrane compartments, such as endosomal/lysosomal membranes, raft, mitochondrial membranes, and endoplasmic membranes. Thus, to understand the in vivo roles of GlcT-1, it is vital to identify precisely the site(s) of ceramide glycosylation. Since GlcT-1 enzymatic activity is detected mainly in the Golgi fraction, GlcT-1 is thought to be primarily located within the Golgi apparatus (17, 18, 20). However, the possibility remains that GlcT-1 may also

**Fig. 1.** GlcT-1 is conserved among various species. The amino acid sequence of DGlcT-1 is aligned with those of human, mouse, C. elegans, the fungus Neurospora crassa, the bacterium cyanobacteria, and plant G. arboreum. All GlcT-1s except for cyanobacteria have enzymic activity. Residues that are conserved in four or more species are indicated in blue; the identical amino acid residues among all species are indicated in red.

**Fig. 2.** DGlcT-1 has GlcCer synthase activity. TLC analysis was carried out with cell lysates. Lanes: 1, MEB-4 cells without UDP-Glc; 2 and 6, MEB-4 cells; 3, GM-95 (GlcT-1-deficient) cells; 4, pcDNA3.1 cells; 5 and 7, DGlcT-1 cells. TLC was performed at 32 °C (lanes 1–5) or 25 °C (lanes 6 and 7).
FIG. 3. Restoration of GSL expression by DGlcT-1. Flow cytometry of MEB-4, GM-95, pcDNA3.1, and DGlcT-1 cells. GM3 was detected in MEB-4 cells and DGlcT-1 cells, but not in GM-95 cells and pcDNA3.1 cells.

FIG. 4. DGlcT-1 mRNA is detectable during all developmental stages in Drosophila. A, Northern blot of mRNAs from various developmental stages of Drosophila revealed a single, 2.3-kb DGlcT-1 transcript. Although it was not detectable in this figure, the GlcT-1 transcript could be detected when the blot was exposed for a long period. Drosophila actin mRNA was used as a control. E, embryo; L, larva; A, adult. B, whole mounts of wild-type embryos of various stages were subjected to in situ hybridization with DGlcT-1 antisense or sense RNA probes.

localize to other intracellular compartments. To address this issue, we examined the distribution of DGlcT-1 in GM-95 cells co-transfected with V5/His-tagged DGlcT-1 (DGlcT-1/V5-His) and pEYFP-Golgi, pEYFP-ER, or pEYFP-Mito, fluorescent markers for Golgi, ER, and mitochondria, respectively. DGlcT-1 was detected immunocytochemically with an antibody directed against the V5 tag, and co-localization with the various organelle markers was analyzed with confocal microscopy. As shown in Fig. 5, dense V5 immunoreactivity (Fig. 5A, red) overlapped precisely with the Golgi marker pEYFP-Golgi (Fig. 5B, green), indicating that DGlcT-1/V5-His localized mainly to the Golgi apparatus (see Fig. 5C, merged image of Fig. 5A and B). In addition, V5 immunoreactivity (Fig. 5D, red) overlapped with the ER marker pEYFP-ER (Fig. 5E, green), indicating that DGlcT-1/V5-His also localized to the ER (see Fig. 5F). By contrast, V5 immunoreactivity (Fig. 5G, red) did not overlap with the mitochondrial marker pEYFP-Mito (Fig. 5H, green; see Fig. 5I), demonstrating that DGlcT-1/V5-His did not localize to mitochondrial membranes. These results suggest that DGlcT-1 is restricted to the Golgi apparatus and ER. We also obtained a similar localization pattern for HGlcT-1 (data not shown).

To verify these double-labeling observations under in vivo conditions, we examined the intracellular distribution of DGlcT-1 in the heads of adult Drosophila flies using immunoelectron microscopy techniques (Fig. 6). Ultrathin sections were stained with an anti-DGlcT-1 antibody directed against amino acid residues 88–101 of DGlcT-1 (Fig. 6A). Western blotting analysis showed that this antibody recognized DGlcT-1 specifically (Fig. 6B). As shown in Fig. 6C, dense DGlcT-1 immunoreactivity localized within the Golgi membranes (Fig. 6C, arrow) as well as within perinuclear ER and ER membranes (Fig. 6C, arrowhead). Very similar results were also obtained when ultrathin sections were stained with anti-DGlcT-1 antibody and a gold-conjugated secondary antibody (data not shown). Consistent with our confocal microscopy findings, electron dense DGlcT-1 immunoreactivity was neither observed in the mitochondria nor plasma membranes. Control serum did not give any positive staining (data not shown).

N-terminal and TM Regions of GlcT-1 Are Required for Golgi Localization—Of the glycosylation enzymes involved in GSL synthesis, GlcT-1 is unique in that it is the only glycosyltransferase that carries out its reactions in the parts of the Golgi apparatus adjacent to the cytoplasm (20–22). Hydropathy plot analysis revealed that GlcT-1 has a single strong hydrophobic segment near its N terminus (amino acids 1–10), a putative signal-anchor sequence containing a single strong hydrophobic segment (amino acids 11–36), and a long cytosolic tail that may be a catalytic domain (15). To determine whether the putative signal anchor sequence is responsible for localizing GlcT-1 to the Golgi, we made V5/His-tagged fusion protein constructs containing truncated forms of HGlcT-1 (Fig. 7I), transfected GM95 cells with these constructs, and then we examined the intracellular distribution of these truncated forms with anti-V5 antibody. As expected, full-length GlcT-1 localized within the
membranes. Immunopositive staining in the perinuclear ER and ER membranes may indicate that both the N-terminal and TM regions (amino acids 1–36) of GlcT-1 are important for Golgi localization.

To confirm whether the N-terminal region alone is sufficient for localization to the Golgi, we made dsRed fusion protein constructs containing the N-terminal region plus the membrane-spanning region (Fig. 7I, panel I), and examined the expression of the construct in GM95 cells. In cells that expressed the dsRed-tagged fusion protein containing both the N-terminal region and the membrane-spanning region, intense fluorescence localized within the Golgi. These deletion analyses indicate that both the N-terminal and TM regions (amino acids 1–36) of GlcT-1 are important for Golgi localization.

Ectopic Expression of GlcT-1 Partially Rescues Cell Death Induced by RPR, GRIM, and HID—To examine the in vivo function of GlcT-1 in Drosophila, UAS-DGlcT-1 lines of flies were crossed with GMR-Gal4 lines, and the eye phenotype of the resulting progeny was examined for signs of apoptosis. Retinal apoptosis is typically manifested by small eye size and/or eye ablations (32–34). Transgenic flies carrying a single copy of UAS-DGlcT-1 (UAS-DGlcT-1/CyO; GMR-Gal4/TM3) did not exhibit significant abnormalities in eye phenotype (Fig. 8B). The fairly normal eye morphology displayed by these flies may be caused by low level expression of DGlcT-1. Flies carrying increased copies of UAS-DGlcT-1 (2 or 3 copies) also did not exhibit eye abnormalities (data not shown). Although we screened a number of UAS-DGlcT-1 lines, we failed to obtain transgenic flies that expressed high levels of DGlcT-1. This was also observed for transgenic mice that overexpress GlcT-1.2 These results suggest that GlcT-1 expression levels might be tightly regulated and high level expression of GlcT-1 might be toxic to cells.

In Drosophila, apoptosis occurs during embryonic development and metamorphosis and is controlled by the expression of three genes, reaper (rpr) (32), grim (33), and head involution defective (hid) (34). Although RPR-induced apoptosis was previously shown to be associated with increased ceramide production that is blocked by an ICE-like protease inhibitor (6), the relationship between ceramide production and GRIM- or HID-induced apoptosis remains unknown.

To assess the role of DGlcT-1 in regulating apoptosis, UAS-dGlcT-1 lines of flies were crossed with GMR-rpr, GMR-grim, or GMR-hid lines. The expression of the cell death activators RPR, GRIM, and HID triggers ectopic apoptosis in retinal cells, which is characteristically manifested by reduced eye size and a partially ablated eye phenotype (Fig. 8, C, E, F, and H) (35). The penetrance of these manifestations is highly sensitive to the number of transgenes present and alterations of the downstream cell death genes (35). If ceramide is involved in the RPR, GRIM-, or HID-induced apoptosis pathways, we would expect that the presence of DGlcT-1 would suppress the eye phenotypes associated with these genes by possibly reducing ceramide levels. As shown in Fig. 7, D and F, in flies harboring DGlcT-1 and either RPR or GRIM, DGlcT-1 overexpression partially suppressed both the RPR- and GRIM-associated reduction in eye size. On the other hand, in flies harboring both DGlcT-1 and HID, DGlcT-1 overexpression failed to clearly affect eye size. Even though these flies still manifested the reduced eye phenotype, few ommatidia were observed (Fig. 8H). No ommatidia were observed in the eyes of flies that expressed only HID (Fig. 8I). These results indicate that DGlcT-1 can function as a negative regulator of cell death mediated by the proapoptotic factors RPR and GRIM.

Inhibition of DGlcT-1 Expression Increases Cell Death—Currently, specific DGlcT-1 mutants are unavailable. Therefore, to further examine the role of DGlcT-1 in apoptosis, we carried out dsRNAi studies to inhibit DGlcT-1 gene function in Drosophila. dsRNAi technologies were originally established in C. elegans (36), but recently these methods have been successfully applied to the study of Drosophila embryogenesis (28, 37). Precellularized embryos were injected with DGlcT-1 dsRNA, grown to midstage, then stained with the TUNEL method to assess apoptosis. As shown in Fig. 9C, DGlcT-1 RNAi increased the number of TUNEL-positive cells in the embryo. Buffer-injected embryos showed no difference in TUNEL staining compared with that of the wild type (CS) (Fig. 9, A and B). For further confirmation of the action specificity of GlcT-1, we cloned the s2(tribluntransformase) (ST6Gal) from Drosophila (29), and then tested whether ST6Gal dsRNA injection induced apoptotic cell death. No enhancement of cell death was observed in the transfected embryos throughout the development. Thus, we concluded that that cell death caused by dsRNAi was specific to DGlcT-1 and the ceramide glycosylation is required for inhibiting cell death during embryogenesis.

**DISCUSSION**

In the present study, we found that DGlcT-1 is highly homologous to the GlcT-1 of other organisms (Fig. 1) and has catalytic activity to form GlcCer (Figs. 2 and 3). Drosophila has no other GlcT-1 homologues. In Drosophila, GlcCer is a major component of detergent-insoluble membranes or lipid rafts (24). Thus, we concluded that DGlcT-1 is the sole enzyme responsible for directing the synthesis of GlcCer in Drosophila. Because GlcT-1 is evolutionarily conserved and GlcCer is distributed widely in eukaryotes, we expect that GlcCer synthesis has basic functions in the cellular machinery. The ubiquitous expression of DGlcT-1 mRNA (Fig. 4) during development also suggests that DGlcT-1 is essential for embryogenesis.

The identity of the site of ceramide production and degradation within a cell is very important for understanding ceramide-mediated signaling mechanisms. Synthesis of ceramide occurs in various cellular compartments. Enzymes involved in ceramide synthetic pathways localize to various compartments; at least seven SMases, which hydrolyze SM to generate ceramidase-mediated signaling mechanisms.
**Fig. 7.** The N-terminal region of GlcT-1 is required for Golgi localization. I, GlcT-1 fusion protein constructs tested for identification of putative Golgi retention signal(s). The horizontal bars represent HGlcT-1 sequences present in the deletion mutants. Bars A–I show a series of truncated V5/His-tagged or dsRED-GlcT-1 fusion proteins. The top part of the figure shows a schematic drawing of intact HGlcT-1. The putative Golgi localization signal region and the transmembrane region are represented by solid and hatched boxes, respectively. The localization and GlcT-1 activities of the fusion proteins are shown in the right panel. II, intracellular localization of HGlcT-1 deletion mutant containing the N-terminal region (1–36 aa) in GM-95 cells. The fusion protein of the construct I localizes to the Golgi apparatus. To determine the intracellular localization, pEYFP-Golgi marker was co-transfected to GM-95 cells.

---

**Fig. 8.** Suppression of RPR- or GRIM-induced cell death by DGlcT-1. Whole mounts of different phenotypes were analyzed by light microscopy (A–H) and scanning electron microscopy (I and J). A, wild-type (CS); B, UAS-DGlcT-1/CyO; GMR-GALA/TM3; C, GMR-RPR/CyO; GMR-GALA/TM3; D, GMR-RPR/UAS-DGlcT-1; GMR-GALA/TM3; E, GMR-GRIM/CyO; GMR-GALA/TM3; F, GMR-GRIM/UAS-DGlcT-1; GMR-GALA/TM3; G and I, GMR-HID/CyO; GMR-GALA/TM3; H and J, GMR-HID/UAS-DGlcT-1; GMR-GALA/TM3. Apoptosis induced by RPR (C), GRIM (E), and HID (G and I) was manifested as a severely roughened, small eye. Co-expression of DGlcT-1 with either RPR or GRIM partially suppressed the small-eye phenotype (D and F). However, HID-induced cell death was not rescued by DGlcT-1 (G–J).

---

Glucoamylose Synthase of D. melanogaster

Ceramide, the GlcT-1-mediated glycosylation of ceramide, is involved in the production of ceramide. Since primary structures of mammalian and *Drosophila* GlcT-1 are conserved from the N terminus to the C terminus, it is quite plausible that both mammalian GlcT-1 and DGlcT-1 localize to the ER as well as to the Golgi. We found that the N-terminal and transmembrane regions (amino acids 1–36) determine HGlcT-1 localization within the Golgi. HGlcT-1 is a type III protein. Its C terminus extends into the cytosol (15), and forms noncovalent dimers or oligomers (40). All truncated forms of HGlcT-1 (Fig. 7A) failed to show enzymatic activity, suggesting that, as with other enzymes, a complete threedimensional structure is probably required for enzymatic activity. The truncated form of HGlcT-1 (Fig. 7A, row E) that lacks a UDP-Glc binding amino acid (histidine 193, Ref. 41) may be incapable of binding the glucose donor.

Functional analysis using transgenic flies and RNAi revealed that DGlcT-1 acts as a negative regulator of apoptosis. DGlcT-1 partially suppressed RPR- and GRIM-mediated apoptosis (Fig. 8), and loss of DGlcT-1 resulted in increased apoptosis (Fig. 9). RPR-induced apoptosis is associated with increased ceramide, the production of which requires an ICE-like protease (6). Nonetheless, these data do not provide adequate evidence to show that ceramide may be a signaling molecule in RPR-induced apoptosis or that it may be formed as a result of apoptosis. Our results, however, clearly indicate that ceramide is a signaling molecule in apoptosis and is also involved in both RPR- and GRIM-induced apoptosis. Interestingly, DGlcT-1 had very little effect on HID-induced apoptosis. This may be caused by, in part, to the fact that pathways downstream of RPR, GRIM, and HID are quite diverse and can be differentially affected by DGlcT-1. For example, expression of dcp-1, an effector caspase, augments the small rough-eye phenotype induced by the ectopic expression of rpr or grim, but not of hid (41). On the other hand, expression of the dominant negative
form of damm, which encodes another effector caspace, suppresses the ablated-eye phenotype resulting from the overexpression of hid, but not rpr (42).

Another possible reason for the differential effects of DGlct-1 on HID-induced apoptosis may be related to the observation that the expression pattern of HID differs from that of RPR and GRIM. RPR and GRIM are solely expressed in cells that undergo apoptosis, but HID is expressed in cells that die and also those that do not die (34). In addition, HID activity is negatively regulated by the EGF receptor pathway (43, 44).

Indeed, the regulation of cell death is very complex, and the pathways that lead to cell death vary depending on the cell type and the stimuli that trigger it. Taken together, these results indicate that the glycan chains in GlcT-1 knockout mice display embryonic lethality (24). Upon examination of these embryos, enhanced apoptosis was detected in the neuroectoderm. This is consistent with our findings in Drosophila in which increased apoptosis was detected in the egghead and brainiac embryos. Nonetheless, our results might help to illuminate the complex mechanisms of cell death.

GlcT-1 knockout mice display embryonic lethality (24). Upon examination of these embryos, enhanced apoptosis is detected in the neuroectoderm. This is consistent with our findings in Drosophila in which increased apoptosis was detected in the egghead and brainiac embryos. Nonetheless, our results might help to illuminate the complex mechanisms of cell death.
Glucosylceramide Synthase of D. melanogaster

47. Muller, R., Altmann, F., Zhou, D., and Hennet, T. (2002) *J. Biol. Chem.* **277**, 32417–32420
48. Goode, S., Melnick, M., Chou, T. B., and Perrimon, N. (1996) *Development* **122**, 3863–3879
49. Goode, S., Morgan, M., Liang, Y. P., and Mahowald, A. P. (1996) *Dev. Biol.* **178**, 35–50
50. De Maria, R., Lenti, L., Malisan, F., d’Agostino, F., Tomassini, B., Zeuner, A., Rippo, M. R., and Testi, R. (1997) *Science* **277**, 1652–1655
51. Rippo, M. R., Malisan, F., Ravagnan, L., Tomassini, B., Condo, I., Costantini, P., Susin, S. A., Rufini, A., Todaro, M., Kroemer, G., and Testi, R. (2000) *Faseb J.* **14**, 2047–2054
52. Schwarz, A., and Futerman, A. H. (1997) *J. Neurosci.* **17**, 2929–2938
53. Adachi-Yamada, T., Gotoh, T., Sugimura, I., Tateno, M., Nishida, Y., Onuki, T., and Date, H. (1999) *Mol. Cell. Biol.* **19**, 7276–7286