Identification of a Molecular Target of Psychosine and Its Role in Globoid Cell Formation

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Abstract. Globoid cell leukodystrophy (GLD; also known as Krabbe’s disease) is a hereditary metabolic disorder of infants, characterized morphologically by apoptosis of oligodendrocytes, progressive demyelination, and the existence of large, multinucleated (globoid) cells derived from perivascular microglia. The glycosphingolipid, psychosine (D-galactosyl-β-1,1’ sphingosine), accumulates to micromolar levels in GLD patients who lack the degradative enzyme galactosyl ceramidase. Here we document that an orphan G protein–coupled receptor, T cell death–associated gene 8, is a specific psychosine receptor. Treatment of cultured cells expressing this receptor with psychosine or structurally related glycosphingolipids results in the formation of globoid, multinuclear cells. Our discovery of a molecular target for psychosine suggests a mechanism for the globoid cell histology characteristic of GLD, provides a tool with which to explore the disjunction of mitosis and cytokinesis in cell cultures, and provides a platform for developing a medicinal chemistry for psychosine.

Key words: psychosine • G protein–coupled receptor • cytokinesis • leukodystrophy • sphingolipid

Introduction

Globoid cell leukodystrophy (GLD; also known as Krabbe’s disease) is a hereditary metabolic disorder of infants, characterized morphologically by almost total absence of myelin, severe gliosis, and the presence of characteristic, multinucleated globoid cells in the white matter (Suzuki and Suzuki, 1978). The deficiency of the catabolic enzyme galatosyl ceramidase results in accumulation of psychosine (PSY; D-galactosyl-β-1,1’-sphingosine) in the brain (Suzuki and Suzuki, 1978). This accumulation of PSY in the white matter of children with GLD correlates temporally with apoptosis of oligodendrocytes and globoid cell formation by microglia (Tanaka and Webster, 1993; Cho et al., 1997). The nature of a causal relationship between globoid cell formation and disappearance of oligodendrocytes, if any, is not understood. The course of the human disease is mimicked by the galactosyl ceramidase (GALC)-deficient twitcher mouse (Igisu and Suzuki, 1984). The homozygous GALC+/GALC− twitcher mice are phenotypically normal at age 22 d but afterwards exhibit head twitching, progressive paralysis, and death by age 45 d (Matsushima et al., 1994). These GALC+/GALC− mice accumulate high levels (120 μM in brain at age 31 d) of PSY (Shinoda et al., 1987).

Although PSY has been long suspected as a molecular agent in GLD and its mouse model, the mechanism of action of PSY is not understood (Suzuki, 1998). Recently, Xu et al. (2000) demonstrated that sphingosylphosphorylcholine (SPC) is a ligand for an “orphan” (i.e., previously unknown ligand) G protein–coupled receptor named ovarian cancer G protein–coupled receptor (OGR1). Due to our long-standing interest in lysosphospholipid mediators such as sphingosine 1-phosphate, we began studying additional orphan G protein–coupled receptors that are similar to OGR1. One of these, named T cell death–associated gene 8 (TDAG8; so named because it is one of the genes expressed to high levels during the programmed cell death of immature T lymphocytes [Choi et al., 1996]), shares 41% identical amino acids with OGR1. In the course of testing a set of putative and known lipid signaling molecules, we found that TDAG8 is a specific receptor for PSY and several related gly-
cogmphingolipids. Further, the PSY/TDAG8 pair evokes a multinuclear phenotype in cultured cells reminiscent of the globoid cell formation that is the neurohistologic fingerprint of Krabbe’s disease.

Materials and Methods

Materials
GluPSY, LaCPSY, N-acetyl PSY, and lysosulfatide were from Matreya, Inc.; PSY and SPC were obtained from Avanti Polar Lipids. [α-32P]CTP was obtained from ICN Biochemicals; pcdNA3 plasmid was from Invitrogen; RH7777 cells (CRL 1601) and HEK293 cells (CRL-1573) were from the American Type Culture Collection; and human multiple tissue expressed 
The human RNA master blot (CLONTECH Laboratories, Inc.) was used. The human RNA master blot (CLONTECH Laboratories, Inc.) was hybridized and washed according to the protocol supplied by the manufacturer.

Northern Blot Analysis
For hybridization, a 32P-labeled human TDAG8 cDNA fragment was used. The human RNA master blot (CLONTECH Laboratories, Inc.) was hybridized and washed according to the protocol supplied by the manufacturer.

DAPI Staining
For DAPI staining, cells were grown on coverslips and treated with 10 μM PSY for 6 (RH7777 cultures) or 4 d (HEK293 cells). After treatment, cells were washed with PBS twice and incubated with cold 70% ethanol for 45 min. Coverslips were then dried and mounted onto slides using Vectashield with DAPI (Vector Laboratories). Confocal microscopy was performed using a Micro Systems LSM (ZEISS) and Axiovert 100 inverted scope at an excitation wavelength of 488 nM with 63X magnification for green fluorescent protein (GFP).

Flow Cytometry
Cells were treated with 10 μM PSY for 6 d, harvested, and then fixed with 70% ethanol. Cells were treated with RNase A (0.1 mg/ml in PBS) at 37°C for 30 min, stained with propidium iodide (50 μg/ml in PBS), and then subjected to flow cytometry with a FACScan™ flow cytometer (Becton Dickinson) for measurement of the DNA content.
Results and Discussion

When expressed in RH7777 hepatoma cells, human TDAG8 mediated PSY-induced inhibition of forskolin-driven cAMP rise in a concentration-dependent manner (EC_{50} = 3.4 μM) (Fig. 1, A and B). This response was evoked also by structurally related lysolipids, e.g., N-acetyl PSY, sphingosine 1-phosphate, lyso-phytosphingosine acid, ceramide 1-phosphate, or lysophosphatidylcholine (Fig. 1 C). Similar results were found using the orthologous mouse TDAG8 DNA (data not shown). The PSY response was not blocked by pretreatment of RH7777 cultures with pertussis toxin (PTX; 10 μg/ml for 24 h), suggesting the involvement of PTX-insensitive G proteins, perhaps G_{a}(Kozasa and Gilman, 1995).

SPC also was active in this assay, but this response, which was PTX sensitive (not shown), probably proceeds through an endogenous receptor in RH7777 cells (Im et al., 2000a). The PSY response was not blocked by pretreatment with syl-glycolipids (e.g., GlcPSY and lysosulfatide), but not by N-acetyl PSY, sphingosine 1-phosphate, lyso-phytosphingosine acid, ceramide 1-phosphate, or lysophosphatidylcholine (Fig. 1 C). Similar results were found using the orthologous mouse TDAG8 DNA (data not shown). The PSY response was not blocked by pretreatment of RH7777 cultures with pertussis toxin (PTX; 10 μg/ml for 24 h), suggesting the involvement of PTX-insensitive G proteins, perhaps G_{a}(Kozasa and Gilman, 1995).

To test the hypothesis that PSY acting via TDAG8 mediates the disjunction of mitogenesis and cytokinesis characteristic of globoid cells, we treated cell cultures transfected with TDAG8 DNA with PSY and quantified nuclear DNA content. When TDAG8-expressing RH7777 cells were treated with 10 μM PSY, multinuclear globoid cells were observed by DAPI staining (Fig. 5 A, and Table I). Neither expression of TDAG8 without PSY treatment nor PSY treatment in the absence of TDAG8 DNA transfection resulted in the appearance of multinuclear, globoid cells. Likewise, TDAG8/HEK293 cells became multinuclear in response to PSY treatment (Fig. 5, B and C) and both receptor and ligand were required to generate the globoid cell phenotype. In concert with the structure activity profile found with inhibition of cAMP and calcium mobilization (see above), GlcPSY and lysosulfatide mimicked the action of PSY in evoking globoid cell formation, whereas N-acetyl PSY and SPC did not (data not shown).

The requirement for both members of the ligand receptor pair to be present, the long time course required for globoid cell formation, and a previous report using U937 cells (Kanazawa et al., 2000) suggest that this phenomenon involves a disjunction of mitosis and cytokinesis rather than simple cell fusion.

Our identification of a PSY receptor and the role of this ligand receptor pair in evoking globoid cell formation has several implications. First, TDAG8 is the second member of the OGR1 receptor cluster found to have a lipid ligand; OGR1 is activated by SPC (but not PSY), whereas two other members of the cluster, G2A (Weng et al., 1998) and GPR4 (Heiber et al., 1995), remain to be paired with a ligand. Our identification of a PSY receptor confirms the prescient observations of Okajima and Kondo (1995) and Himmel et al. (1998), both of whom suggested that PSY acts through a specific G protein–coupled receptor. Second, the PSY receptor, presumably acting through un-

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Multinuclear cells (mean ± SEM)</th>
<th>Multinuclear cells/total cell count</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.18 ± 0.48</td>
<td>32/1,437 (3)</td>
</tr>
<tr>
<td>PSY</td>
<td>39.32 ± 3.38</td>
<td>378/984 (6)</td>
</tr>
<tr>
<td>GlcPSY</td>
<td>44.08 ± 4.08</td>
<td>392/890 (6)</td>
</tr>
<tr>
<td>Lysosulfatide</td>
<td>21.16 ± 3.24</td>
<td>243/1,138 (3)</td>
</tr>
<tr>
<td>LacPSY</td>
<td>4.10 ± 0.29</td>
<td>67/1,617 (3)</td>
</tr>
<tr>
<td>SPC</td>
<td>6.27 ± 0.74</td>
<td>57/900 (3)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses indicate total number of experiments.

Table I. PSY Induces Multinuclear Cells in TDAG8-expressing RH7777 Cultures

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known heterotrimeric G proteins, blocks cell division, but not nuclear division, and thus provides a tool that might be useful in exploring mechanisms of cytokinesis. It is noteworthy that two tissues that contain multinuclear cells, placenta (trophoblasts) and lung (macrophages), are prominent in expressing TDAG8 RNA (Fig. 4). Third, the structure activity profile of PSY receptor ligands suggests that TDAG8 might be involved in the pathogenesis of related lipid storage disorders, such as Gaucher’s disease (accumulation of GlcPSY), which is characterized by hepatomegaly, splenomegaly, and osteoporotic erosion (Brady, 1978), and metachromatic leukodystrophy (lysosulfatide accumulation), which is characterized by myelin degeneration (Moser and Dulaney, 1978). However, neither of these diseases is associated with the globoid cell formation characteristic of Krabbe’s disease. Finally, the identification of a molecular target for PSY and related lipids provides a platform on which a medicinal chemistry, including the discovery and optimization of receptor blockers, can be built. PSY antagonists might prove useful clinically in altering the course of some lipid storage disorders. Currently, only palliative care is available to
Krabbe’s disease and metachromatic leukodystrophy patients, whereas patients suffering from the often more indolent Gaucher’s disease have available enzyme replacement therapy (albeit at enormous cost). The twitcher mouse provides a convenient model for testing potential receptor blockers. In addition, if a mouse with its TDAG8 genes ablated proves fertile, crossing this genotype onto a twitcher background could prove informative.

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References

Figure 5. Inhibition of cytokinesis by PSY. TDAG8-RH7777 (A) or TDAG8-HEK293 (B) cells were stained with DAPI (middle). Green lines on overlays indicate boundaries of single cells. (C) Representative FACS® data from HEK293 cells (top) or HEK293 cells expressing TDAG8 (bottom) treated either with 10 μM PSY (C-2 and C-4) or vehicle (C-1 and C-3). Cell cultures were treated for 6 d.


