Triacylglycerol mimetics regulate membrane interactions of glycogen branching enzyme: implications for therapy

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Abstract Adult polyglucosan body disease (APBD) is a neurological disorder characterized by adult-onset neurogenic bladder, spasticity, weakness, and sensory loss. The disease is caused by aberrant glycogen branching enzyme (GBE) (GBE1Y329S) yielding less branched, globular, and soluble glycogen, which tends to aggregate. We explore here whether, despite being a soluble enzyme, GBE1 activity is regulated by protein-membrane interactions. Because soluble proteins can contact a wide variety of cell membranes, we investigated the interactions of purified WT and GBE1Y329S proteins with different types of model membranes (liposomes). Interestingly, both triheptanoin and some triacylglycerol mimetics (TGMs) we have designed (TGM0 and TGM5) markedly enhance GBE1Y329S activity, possibly enough for reversing APBD symptoms. We show that the GBE1Y329S mutation exposes a hydrophobic amino acid stretch, which can either stabilize and enhance or alternatively, reduce the enzyme activity via alteration of protein-membrane interactions. Additionally, we found that WT, but not Y329S, GBE1 activity is modulated by Ca2+ and phosphatidyserine, probably associated with GBE1-mediated regulation of energy consumption and storage. The thermal stabilization and increase in GBE1Y329S activity induced by TGM5 and its omega-3 oil suggest that this molecule has a considerable therapeutic potential for treating APBD.—Alvarez, R., J. Casas, D. J. López, M. Ibarguren, A. Sauri-Rivera, S. Terés, F. Guardiola-Serrano, A. Lossoś, X. Busquets, O. Kakhlon, and P. V. Escribá. Triacylglycerol mimetics regulate membrane interactions of glycogen branching enzyme: implications for therapy. J. Lipid Res. 2017. 58: 1598–1612.

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Abbreviations: APBD, adult polyglucosan body disease; Cho, cholesterol; DSC, differential scanning calorimetry; DSF, differential scanning fluorimetry; ER, endoplasmic reticulum; GBE, glycogen branching enzyme; GYS, glycogen synthase; IOD, integrated optical density; LUV, large unilamellar vesicle; Ni-NTA, nickel-nitrilotriacetic acid; PBMC, peripheral blood mononuclear cell; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI(4,5)P2, phosphatidylinositol-4,5-bisphosphate; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; PS, phosphatidyserine; TGM, triacylglycerol mimetic; TH, triheptanoin; Tm, melting temperature.

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The glycogen branching enzyme (GBE1) (EC 2.4.1.18) catalyzes two reactions. In amyylase-type hydrolysis, GBE1 cleaves an α-1,4-linked segment of six or more glucose units, every 8–14 glucose residues from the nonreducing end of a glucon chain. In the transglucosylation reaction, it transfers the cleaved oligosaccharide, via an α-1,6-glucosidic linkage, to the C6 hydroxyl group of a glucose acceptor unit within the same chain (intramolecular) or onto a different neighboring chain (intermolecular (1)). The resulting glycogen is a highly branched molecule whose spherical and soluble structure favors its cytoplasmic stability and metabolism (2). A mutant GBE1 enzyme may affect the structure of glycogen, reducing its cytoplasmic solubility and hindering its bio-availability due to the formation of aggregates called polyglucosans. Different mutations partially or completely inactivate GBE1, and the severity of the pathophysiological symptoms can vary as a function of the mutation (3–6).

Adult polyglucosan body disease (APBD) is a rare neurodegenerative disease that most often affects adults from the Ashkenazi Jewish community afflicted by the substitution of tyrosine-329 by a serine residue, which impairs GBE1 activity. Inactivation of GBE1 and the activation of glycogen synthase (GYS) leads to the generation of polyglucosan bodies (amylopectin-like polysaccharides with fewer branch points), which are harmful to neurons and glial cells. These aggregates may cause different phenotypic alterations, such as neurogenic bladder, partial motor dysfunction in the extremities, sensorial dysfunction in the lower part of the body, and storage.

The thermal stabilization and increase in GBE1Y329S activity induced by TGM5 and its omega-3 oil suggest that this molecule has a considerable therapeutic potential for treating APBD.
body, and possibly cognitive impairment. The advanced state of the disease is characterized by difficulty in walking, impaired balance, and progressive weakness, sometimes accompanied by early death (4, 5). Triheptanoin (TH; a triacylglycerol with three heptanoic acid moieties) is currently under investigation as a possible treatment for APBD [ClinicalTrials.gov identifier NCT00947960 (7, 8)]. However, little is known about the molecular events underlying the effects of TH and its possible clinical efficacy is still unclear. Thus, this drug remains far from achieving marketing approval from the major regulatory agencies (e.g., European Medicines Agency, Food and Drug Administration, etc.).

Most processes in the cell occur in, at, or around membranes (9). The high density and the variety of cell membranes in the cytoplasm, with different compositions and physicochemical properties [i.e.: the plasma membrane; the mitochondrial, nuclear, lysosomal, endosomal, peroxysomal, Golgi, and vesicular trafficking membranes; the endoplasmic reticulum (ER); and other types of vesicular and organellar membranes in different cell types] favors the interactions of soluble proteins with different lipid bilayers. Because the p.Y329S GBE1 mutation causes partial misfolding that may expose internal hydrophobic regions of the enzyme to the aqueous milieu, the activity of this enzyme may be modulated by GBE1-membrane interactions. Such interactions would be influenced by changes in membrane lipid composition (1). Indeed, the therapeutic effects of the lipid TH suggest that membrane lipids may play a relevant role in the etiology, pathophysiology, and therapy of APBD. As such, the present study was designed to investigate the interactions of WT and p.Y329S-mutated GBE1 with model membranes, so as to study the molecular bases underlying APBD. In addition, we set out to design and investigate molecules that could regulate this interaction, enhancing GBE1 activity, in the hope of defining possible therapeutic tools for the treatment of this condition.

MATERIALS AND METHODS

Materials

The pFastBac 1 plasmid and SYPRO-Orange were purchased from Invitrogen (Barcelona, Spain), E.coli and Nov were obtained from Fermentas (Madrid, Spain), while shrimp alkaline phosphatase was obtained from USB Corporation (Staufen, Germany). Agarose D-1 was obtained from Conda Laboratories (Barcelona, Spain) and Grace’s medium was from GIBCO (Madrid, Spain). DTT, HEPES, phosphorylase-a, Tris-HCl, proteinase inhibitors, and all other reagents were purchased from Sigma-Aldrich (Madrid, Spain).

TH and the triacylglycerol mimetics (TGMs) were produced by BEGA Pharmaceuticals (Palma de Mallorca, Spain). The TGMs used here are triacylglycerols with three identical hydroxylated fatty acid molecules, such that: TGM0 is the hydroxylated analog of TH and it contains three 2-hydroxy-heptanoic acid moieties; TGM1 has three 2-hydroxy-oleic acid moieties; TGM2 has 2-hydroxy-linoleic acid moieties; TGM3A has 2-hydroxy-arachidonic acid moieties; TGM3G has 2-hydroxy-eicosapentaenoic acid moieties; and finally, TGM6 has 2-hydroxy-docosahexaenoic acid moieties. The number in the TGM abbreviation corresponds to the number of double bonds in the fatty acid molecule.

GBE1 cloning and site-directed mutagenesis

The cDNA encoding the recombinant human GBE1 protein (GenBank accession number BC012098) was kindly provided in the pOTB7 expression vector (1.8 kb) by Dr. Hasan O. Akman (Columbia University, NY). This cDNA was subcloned into the pFastBac-1 vector, adding a C-terminal 6xHis-tag by PCR amplification with the Pfu DNA polymerase (Thermo Scientific, Schwerte, Germany) and using primers with the appropriate sequence (Fig. 1, Table 1). After an initial incubation at 94°C for 5 min, the PCR mixture was subjected to 35 cycles (denaturation at 94°C for 45 s, annealing at 65°C for 45 s, and elongation at 72°C for 2 min), followed by a final 10 min elongation step at 72°C. Human GBE1Y329S (Fig. 1) was generated from the recombinant pFast-Bac-1 plasmid by PCR amplification using primers that contained the selected mutation (Table 1). As such, overlap-extension PCR was carried out in two consecutive reactions using a high-fidelity DNA polymerase (Exact Polymerase; 5 Prime Co., Hilden, Germany). In the first reaction, an initial 2 min incubation at 94°C was followed by 32 thermal cycles (denaturation at 94°C for 45 s, annealing at 68°C for 45 s, and elongation at 72°C for 2 min) and a final 10 min elongation step at 72°C. In this first PCR, two independent reactions were set up, using the “GBE1 forward” and “internal reverse” primers in one of the reactions, and in the second reaction the “internal forward” and “GBE1 reverse” primers. The products of both of these reactions were combined for a second amplification process under the same conditions without the addition of further reagents. The amplicon resulting from this last reaction contained the modifications shown in Fig. 1. Subsequent reactions of enzymatic digestion and ligation, as well as bacterial transformation and purification of the resultant recombinant constructs were done as described elsewhere (10).

GBE1 protein purification

Recombinant GBE1 proteins (WT and Y329S) were produced in S9 cells cultured in suspension in Grace’s medium supplemented with 10% FCS (v/v), penicillin (100 units/ml), and streptomycin (100 µg/ml). The proteins were purified as described elsewhere with slight modifications (11). Briefly, the GBE1 proteins (Fig. 1) were overexpressed and purified from the cytosolic fraction of infected S9 cells after harvesting the cells by centrifugation and resuspending them in 6 ml of ice-cold 20 mM HEPES buffer (pH 8.0) containing β-mercaptoethanol (10 mM), NaCl (100 mM), and proteinase inhibitors (lysis buffer). A final wash of the nickel-nitritolriacetic acid (Ni-NTA) affinity column was done with 12 ml of HEPES buffer (20 mM, pH 8.0), containing MgCl2 (0.5 mM), β-mercaptoethanol (10 mM), NaCl (100 mM), leupeptin (0.5 µg/ml), and imidazole (15 mM) at 30°C. Finally, the GBE1 proteins were eluted with HEPES buffer (20 mM, pH 8.0)
containing β-mercaptoethanol (10 mM), NaCl (100 mM), and MgCl₂ (1 mM, elution buffer) and supplemented with imidazole in a step gradient (20, 40, 80, 120, 240, and 300 mM). The purified protein was desalted, filtered, concentrated with Amicon Ultra-15 (30 kDa) and stored at -80°C. The WT protein produced had good activity, whereas the activity of GBE1Y329S was always less than that of the WT form. In all cases, the activity of GBE1 increased in the presence of PC membranes.

Preparation of model membranes

Model membranes (liposomes) were prepared from 10 mM stock solutions of natural lipids (PC, PE, PS, Cho, and SM) and synthetic triacylglycerols by mixing the appropriate volumes of each in glass vials. The solvent (chloroform:methanol, 2:1, v/v) was then removed under argon and the lipid film was subjected to vacuum for 2 h to remove traces of solvent. Subsequently, the lipid films were resuspended by vigorous vortexing in binding buffer [20 mM HEPES buffer, 100 mM KCl, and 0.1 mM EDTA (pH 7.4)] to a final concentration of 3 mM (lipid phosphorus). These multilamellar vesicles were submitted to 10 freeze/thaw cycles (-196°C/42°C) and, subsequently, the different lipid emulsions were passed 11 times through a 400 nm pore polycarbonate membrane to generate large unilamellar vesicles (LUVs) using a mini-extruder (Avanti Polar Lipids). The final concentration of the liposomes was determined as described elsewhere (10). LUVs were prepared in the presence of calcium following an identical protocol except that 2 mM CaCl₂ was added to the binding buffer. For activity assays, unilamellar vesicles of PC and PC:TGM (4:1) were prepared in activity buffer (20 mM MES buffer, pH 6.3) containing 54 mM glucose-1-phosphate and 2.2 mM AMP.

GBE1 binding to model membranes

Liposomes (1 mM) were incubated for 1 h at 25°C in the presence of 100 ng of purified GBE1 in a total volume of 300 μl binding buffer. Membrane-bound GBE1 was then differentiated from unbound GBE1 by centrifugation at 90,000 g for 1 h at 25°C. Finally, the membrane pellets were resuspended in 36 μl of 80 mM Tris-HCl buffer (pH 6.8) containing 4% SDS, and combined with 4 μl of 10× electrophoresis loading buffer [120 mM Tris-HCl (pH 6.8), 1.43 M β-mercaptoethanol, 2% SDS, and 50% glycerol]. In binding assays with calcium, 2 mM CaCl₂ was added to the binding buffer.

GBE1 protein binding quantification

Binding of WT and mutant GBE1 proteins to model membranes was quantified as described elsewhere (11). Briefly, proteins present in the membrane pellets were fractionated on 9% polyacrylamide gels and then transferred to nitrocellulose membranes. The membranes were then incubated for 1 h at room temperature with gentle rocking in blocking solution containing PBS and 5% (w/v) nonfat dry milk. After blocking, the membranes were probed overnight at 4°C with anti-GBE1 (diluted 1:750 in fresh blocking solution containing 0.1% Tween 20), washed with PBS, and then incubated at room temperature for 1 h with IRDye 800CW-linked donkey anti-mouse IgG (diluted 1:4,000 in blocking solution containing 0.01% SDS). Antobody binding was detected by near infrared fluorescence using an Odyssey near infrared radiation detection system (LI-COR Biosciences). GBE1 protein was quantified using standard curves [i.e., a plot of the GBE1 protein loaded against the integrated optical density (IOD) of the immunoreactive bands], consisting of four points of different protein content prepared from a commercial

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**TABLE 1. PCR primers used for WT and GBE1Y329S amplification**

| Primer Type          | Primer Sequence                          |
|----------------------|------------------------------------------|
| MAAPMTFAAR           | PDEYEALNA ALADVPELAR LLEIDPILKP YAVDPQRRYK QFSQIKNIG |
| EMEGIDFRS            | NRGSVPGRSH CADDGLYCE WAGEARGVFL TGDPMGRNBF SYFYYKLVDG |
| KXELVPPRQ            | NKSVLVPRDS KLXVITSKS CRILYIRPSW AKVYVRGCDM UNYQIWIMDP |
| REVEKREVR            | PPKPRPRLKTY ESYVGISHE QGKVSAYKFT CNWLRIRGQ CYNQCIQLAI |
| MHAYTASFQ            | YQITSPFAAS SRYGTPEILQ ELYPTAHSMEG IIVLLYVHS NASKNSADGL |
| MFOTGATCTG          | FHSGFATGHED LWSKLFAYSA SNEVLRFLIS KINWLEER XFGDFRVDVT |
| SNLHSSGQQ            | QGQFDSTIEY FULQVDRAD TILMLANELV HTLICYSIDT AEVDVMVAPL |
| CIPIORQGGG           | YFDYLMAIP DWNQLKKEP KDREDNMMDI VYTTLNRELV KECIAYAAS |
| QXAJGQDGVR           | APWLMADMAY TMSNLVPTFT PVFIRGQIQH KMRITLTHGL GGBSYPNGF |
| NIFCGHPEWLD          | FPFCGNNESY HAYRQPHLT DDDLRYKRYL NNFPEDEMRL EREYCGLAP |
| QATVNYEERG           | NR1IAFPERAG LLFIFNHFEK KSYYDVFVAT ALGFVFLVDL DSADAMEGCH |
| QRLEHSTDFF           | SEAFEHNGRP SIVLLYIPS RVALIQVNDL FHHHHHH |

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*EcoRI restriction enzyme site underlined.

*Not restriction enzyme site underlined.

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**Fig. 1.** Human GBE1 and GBE1Y329S amino acid sequences. The one-letter code amino acid sequence for the WT GBE1 used in the present study. A C-terminal 6xHis tag was added for purification and immunodetection purposes. In red, the Y-to-S alteration found in APBD patients is shown.
GBE1 protein batch (OriGene). This commercial GBE1 protein was used only for quantification purposes. A linear relationship between the amount of protein loaded onto the gel and the IOD was found over the entire range of protein used. The amount of GBE1 protein in the experimental samples was obtained by interpolation of the IOD value from these samples in the standard curve. Thus, the amount of GBE1 protein bound to LUVs was expressed as the proportion of protein in the pellet fraction with respect to the total quantity of GBE1 initially used in the binding experiment (100 ng).

GBE1 activity

Model membrane suspensions (34 μl containing 6 mM total lipid) and 4 μl of GBE1 (10 ng) were incubated for 1 h at 25°C with shaking at 300 rpm. Enzyme activity was then analyzed at 37°C by determining the glucose-1-phosphate incorporation into glycogen in the absence of the exogenous glycogen primer, as described elsewhere (12). Briefly, 1.39 μl of activity buffer, 2 μl of [14C]glucose-1-P (0.02 mCi/ml), and 25 μl of total boiled rat muscle 10% homogenate were added to each sample, and a 50 μl aliquot of the reaction mixture was analyzed as the baseline (time point 0). Subsequently, 5 μl of rabbit muscle phosphorylase-a (22 μg/μl) were added to the reaction medium and 50 μl aliquots were taken after 30 and 60 min. In assays with calcium, 2 mM CaCl2 were added to the activity buffer. All these samples were then spotted onto filter paper, dried for 3 min, and washed twice with 66% ethanol and once with acetone for 5 min. Finally, the filter papers were dried and 4 ml of scintillation liquid were added to the filter paper in each bottle. The radioactivity was measured by a scintillation counter, measuring the total radioactivity in nonboiled samples and the nonspecific incorporation in preboiled samples. Specific activity was determined as the difference between the total and nonspecific radioactivity.

Differential scanning fluorimetry

Melting temperatures (Tm) for WT and GBE1Y329S proteins were determined in strip-tubes using a StepOnePlus RT-PCR thermal cycler (Life Technologies Corporation). Each sample (20 μl) consisted of purified GBE1 protein (0.5 μM) in 20 mM HEPES (pH 8.0), 100 mM NaCl, 1 mM MgCl2, 0.5 mM EDTA, 1 mM DTT, and 5× SYPRO-Orange (1:1,000 dilution from the original stock). The effects of PC membranes (LUVs) were analyzed in the presence or absence of 20 mol% of the different triacylglycerols, TH, TGM0, or TGM5. A concentration of 12.5 μM of total lipid was used in these assays with model membranes. Fluorescence intensities were measured and they were obtained using the software provided by the manufacturer (TA Universal Analysis).

Data analysis

Origin software was used for the data and statistical analysis. Unless otherwise indicated, the results are expressed as the mean ± SEM from the number of experiments indicated (n). To determine statistical significance, an ANOVA or an unpaired two-sample t-test was used when appropriate. Differences were considered statistically significant at P < 0.05.

RESULTS

The p.Y329S mutation dramatically alters GBE1’s membrane interactions

Recombinant human WT GBE1 protein and its mutant counterpart (GBE1Y329S) were purified by affinity chromatography (Fig. 2) and their interactions with the most representative membrane lipids were analyzed. There is considerable evidence that the p.Y329S mutation introduces important structural changes in GBE1 (1) and,

Triacylglycerol mimetics regulate GBE1 membrane interaction 1601
indeed, this mutant protein had a different Ni-NTA affinity elution profile (Fig. 2). In addition, more mutant GBE1Y329S bound to LUVs (Fig. 3), suggesting that the internal hydrophobic region flanking Y329S could be exposed at the protein’s surface and become involved in membrane lipid interactions, along with other domains. In fact, the p.Y329S mutation drastically altered the binding of GBE1 to PC, PC:PE, PC:PS, PC:PE:Cho:SM, and PC:PE:PS:Cho:SM membranes (Fig. 3, supplemental Fig. S1).

**TGMs regulate GBE1Y329S-membrane interactions**

TH is currently undergoing clinical trials to investigate its efficacy to treat APBD and other conditions (ClinicalTrials.gov identifiers #NCT00947960 and #NCT01993186). For this reason, we designed and synthesized a number of TH analogs (TGMs) in order to obtain potentially improved therapeutic tools. We then investigated the effects of TH and the aforementioned TGMs on the membrane interactions and activity of GBE1 (WT and Y329S). TH and its closest analog, TGM0, were seen to induce a significant concentration-dependent reduction in the binding of GBE1Y329S to PC membranes (Fig. 4A), whereas they failed to affect the binding of WT GBE1 to PC membranes (Fig. 4A). When the effect of the other TGMs on GBE1-membrane interactions was studied, they again conditioned the binding of GBE1Y329S to PC membranes, but not the interaction of WT GBE1. Hence, it would appear that hydrophobic amino acids that may interact with cell membranes become exposed in the mutant enzyme (Figs. 4B, 10; supplemental Fig. S1). In this context, the highly unsaturated TGMs, TGM4, TGM5, and TGM6 (with four, five, and six double bonds per acyl chain, respectively) induced a marked decrease in the binding of GBE1Y329S to PC membranes (Fig. 4B).

**TH, TGM0, and TGM5 enhance GBE1 activity**

Purified WT GBE1 is more active than GBE1Y329S (Fig. 5A), and the presence of PC had a positive effect on the activity of WT GBE1, which was defined as 100%. In the absence of membranes, the activity of WT GBE1 was significantly weaker (approximately 35%). The increase in GBE1Y329S activity induced by TH in PC membranes was in agreement with its potential therapeutic effect (Fig. 5B). Moreover, TGM0, a hydroxylated TH analog, and TGM5 both enhanced GBE1Y329S activity more than TH, suggesting that they could be used to treat APBD, and the in vitro and ex vivo activity of these compounds was evident (Figs. 5A, B; 8). Thus, TGM5 (22% of the WT activity) and TGM0 (28%) induced a marked increase (approximately 2-fold) in activity of GBE1Y329S in the presence of PC alone (13%; Fig. 5B).

An interesting relationship between GBE1 binding to lipid membranes and GBE1 activity was observed, and the strongest enzyme activity appeared to be induced within a discrete range of GBE1Y329S binding to membranes, with more or less binding reducing the enzyme’s activity. (Fig. 5C, E). Outside of this range, more or less GBE1Y329S binding to membranes was associated with weaker catalytic activity. Hence, membrane lipids appear to regulate the activity of GBE1 and consequently, they will regulate glycogen metabolism and storage, as discussed below.
Triacylglycerol mimetics regulate GBE1 membrane interaction

The Tm\ [+the equilibrium point where folded and unfolded protein concentrations are identical according to a reversible two-stage unfolding model (13)] + of GBE1 (48.9°C) was markedly higher than that of GBE1Y329S (41.68°C) (Fig. 6, supplemental Fig. S3), indicating that the mutant protein had lower thermal stability, possibly due to folding alterations. The presence of PC membranes increased the thermal stability of both GBE1 and GBE1Y329S relative to the purified enzyme (Fig. 6). Moreover, PC membranes containing TGM5 (Fig. 6, supplemental Fig. S2, supplemental Table S1), as well as TGM5, added directly to GBE1Y329S without PC membranes (Fig. 7), markedly induced higher Tm values in GBE1Y329S, as determined by differential scanning fluorimetry (DSF). This thermal stabilization of GBE1Y329S was not higher in the presence of TH or TGM0, as compared with PC alone (Fig. 6H, supplemental Fig. S2, supplemental Table S1). Similarly, TGMs induced only modest changes in the thermal stability of WT GBE1 with respect to PC alone (Fig. 6D, supplemental Fig. S2, supplemental Table S1).

TH, TGM0, and TGM5 increased GBE1Y329S activity in APBD PBMCs

In agreement with the above in vitro data, TH, TGM0, and TGM5 also increased GBE1Y329S activity in cells (PBMCs) from APBD patients ex vivo (Fig. 8). Therefore, to determine the potential clinical value of these compounds, we compared the effect of TH, TGM0, and TGM5 in cells (all three activators of GBE1Y329S in vitro) with that of the negative control, TGM1. Overnight incubation of APBD PBMCs with TGM0 and TGM5 significantly enhanced GBE1Y329S activity (Fig. 8), whereas incubation with TH did not significantly affect the enzyme’s activity and TGM1 abolished GBE1Y329S activity. This data further supports the critical influence of membrane structure on this enzyme.

TGMs regulate membrane lipid structure

The effects of the different TGMs on membrane lipid structure were investigated by DSC (Table 2, Fig. 8), analyzing the gel-to-fluid (Tm), and the lamellar-to-hexagonal HII (T_HII) transition temperatures and enthalpies of POPE in the presence or absence of the various TGMs studied here (1 mol%). In this context, TGM0 induced a modest (approximately 1°C) reduction in the Tm values, while the rest of the TGMs failed to produce a significant change (Table 2). Hence, these molecules had little effect on membrane fluidity. Similarly, TGM0 induced a modest reduction in T_HII (approximately 1°C), whereas TGM5 and the other longer unsaturated TGMs induced larger reductions in the transition to the nonlamellar phase (HII). As such, TGMs appear to reduce membrane surface packing, which may allow other hydrophobic protein moieties (lipid or peptide moieties) to penetrate the lipid bilayer.

Ca2+ and PS differentially regulate WT and GBE1Y329S

PS did not have a significant effect on GBE1 binding to PC membranes (Figs. 3, 9), but Ca2+ and PS together drastically changed this behavior. The binding of WT GBE1 to PC:PS was significantly enhanced in the presence of calcium ions (Fig. 9A). However, other important anionic lipids, like PI(4,5)P2, did not affect the binding of WT GBE1 to PC membranes in the presence of Ca2+. This enhanced GBE1-Ca2+-PS interaction was associated with weaker enzymatic activity (Fig. 9B, D), and PI(4,5)P2 also decreased GBE1 activity, albeit to a lower extent than PS (Fig. 9B, D). Thus, important second messengers, like Ca2+, and the membrane lipid, PS, seem to jointly play a key regulatory role in glycogen metabolism.

Fig. 4. Binding of WT GBE1 and GBE1Y329S to PC membranes with different triacylglycerol compositions. A: Binding of WT GBE1 and GBE1Y329S to PC membranes with increasing concentrations of TH and TGM0 (5, 10, and 20 mol%). B: Binding of WT GBE1 and GBE1Y329S to PC membranes in the presence or absence of various TGMs (20 mol%). The bars show the mean proportion of each GBE1 protein bound to model membranes (bound GBE1 protein relative to the total GBE1 protein) and representative immunoblots of each binding experiment are also shown. In all cases the data represent the mean ± SEM of from three to seven independent experiments. **P < 0.01. #Indicates a significant difference in the binding of GBE1Y329S with respect to WT GBE1 (P < 0.05).
GBE1Y329S did not behave like the WT enzyme, and, in the absence of Ca\(^{2+}\), the binding of the mutated protein to PC, PC:PS, and PC:PI(4,5)P\(_2\) membranes was enhanced relative to the WT GBE1 (Fig. 9A, C). In contrast to GBE1, GBE1Y329S binding to PC:PS and PC:PIP\(_2\) membranes was not significantly different in the presence of Ca\(^{2+}\) (Fig. 9C). Finally, Ca\(^{2+}\) and PS or PI(4,5)P\(_2\) induced some reduction in GBE1Y329S activity (Fig. 9B, D).

**DISCUSSION**

Cell membranes play critical roles in the modulation of both soluble and membrane-associated enzymes (11, 17–23). Indeed, it is estimated that over 90% of the activities in a cell occur at or around membranes, in part owing to their huge surface area within cells (24). In this context, part of the cell’s glycogen is degraded in membrane-bound lysosomes by acid α-1,4-glucosidase and, moreover, glycogen is transported to lysosomes via autophagosomes that fuse with their membranes (24). Important glycogen-related enzymes seem to be associated with membranes, such as laforin or GM [glycogen targeting subunit M (25, 26)], the former being a purported regulator of autophagy (27) thought to play an important role in controlling selective glycogen recycling during autophagy (28). Moreover, a physical association between newly formed polyglucans and the ER has been suggested in Lafora disease (29).

As cell membranes are expected to partake in glycogen metabolism and as the synthetic lipid, TH, is already in clinical trials for treating the glycogen storage disorder, APBD, we opted to investigate the interactions of the enzyme mutated in APBD, GBE1, with lipid bilayers, and the effect of membrane lipids on the activity of WT and mutant GBE1. Model lipid bilayers that resemble different membrane types and distinct lipid microdomains in cell membranes were used to determine the effect of cell membranes on GBE1 activity, studying the in vitro interactions of...
Fig. 6. Effect of TH, TGM0, and TGM5 on the thermal stability of WT GBE1 and GBE1Y329S. Changes in $T_m$ (melting temperature that defines the thermal stability) of WT GBE1 (A–D) and GBE1Y329S (E–H) proteins were determined by DSF. These representative graphs show the absolute (dotted line) and relative (continuous line) fluorescence and the increase in $T_m$ (bar graphs) induced by PC membranes in the presence or absence of TH, TGM0, or TGM5 (20 mol%).
purified WT and mutant GBE1Y329S. In addition, we assessed the effect of TH and other triacylglycerols on GBE1-membrane interactions and on the WT and GBE1Y329S enzyme activity. We observed that GBE1Y329S binding to model membranes was enhanced relative to the WT GBE1, possibly due to a conformational change that affected the mutant protein’s hydrophobicity to some extent, but mainly externalized a hydrophobic amino acid stretch in the vicinity of S329 (Fig. 10, Table 3).

Binding of both our GBE1Y329S and the commercial GBE1 to membranes was enhanced relative to our WT GBE1, suggesting misfolding of the former proteins (supplemental Fig. S1). GBE1Y329S misfolding was further suggested by the distinct elution profiles of the GBE1Y329S mutant and the WT GBE1 (Fig. 2), by the lower thermal stability (T_m) of the mutant protein (Fig. 6), by the higher DSF fluorescence level associated with the mutated form (Fig. 6), and by the surface probability close to residue 329 in both the WT and mutant GBE1 (Fig. 10). In general, the binding of GBE1Y329S to membranes had a great impact on its activity. Either little or high protein binding resulted in less enzyme activity, with a peak of GBE1Y329S activity when binding was in the range of 200–250% of that of WT GBE1 to PC (Fig. 5). Hence, lipids may provide protection and can stabilize GBE1Y329S, yet excessive membrane binding may hinder the active site or impose structural changes that could dampen its activity. In this context, the high density of membranes inside cells suggests that GBE1Y329S activity in vivo could be positively or negatively influenced by the membrane lipid composition, which in turn may be modulated by diet or pharmacotherapy (30–33). Thus, although the activity of GBE1Y329S was weaker than that of the WT, both enzymes were more active in the presence of PC membranes. There is a relationship between the p.Y329S mutation, protein hydrophobicity and surface probability (Fig. 10), and the impact of these alterations on
Triacylglycerol mimetics regulate GBE1 membrane interaction

According to this scenario, TH may potentially have therapeutic activity for treating APBD (7), which further supports a possible role for membranes in the regulation of GBE1Y329S and in APBD therapy (31). TH has been proposed to ameliorate APBD by providing an alternative ketogenic source of energy (7). However, polyglucosan bodies clog axons (34) and cause neuronal death by apoptosis (28), indicating that an energetic deficit is not a primary pathogenic driver in APBD, where the exclusion of glycogen from neurons causes it to become a marginal energy source anyway (35). Therefore, using a triacylglycerol as an alternative source of energy to glucose/glucan would not be expected to counteract neurotoxicity. As such, explaining the therapeutic effects of TH in terms of GBE1Y329S stabilization rather than as a means of surmounting the energetic deficit is a novel notion worthy of being pursued.

This hypothesis led us to design a series of triacylglycerols that we called TGMs and that are structurally similar to TH.

**Fig. 8.** Structure and effects of TH, and of TGM0, TGM 1, and TGM 5 on GBE1Y329S activity in APBD patient’s PBMCs. Upper panel: APBD PBMCs were incubated overnight in the presence or absence (APBD) of 300 μM of the triacylglycerides indicated. After harvesting the PBMCs, GBE1Y329S activity was determined as described [mean ± SEM; *P < 0.05 (12)]. Lower panel: Structure of TGM0 and TGM5.

**TABLE 2.** Effect of TGMs (1 mol%) on the POPE transition enthalpies and temperatures

|             | Tm (°C) | ΔH (kcal/mol) | Tm (°C) | ΔH (kcal/mol) |
|-------------|---------|---------------|---------|---------------|
| POPE        | 23.6 ± 0.2 | 4.42 ± 0.03   | 69.1 ± 0.5 | 0.42 ± 0.03   |
| TH          | 23.1 ± 0.2 | 5.90 ± 0.22   | 62.5 ± 0.7 | 0.50 ± 0.06   |
| TGM0        | 22.7 ± 0.2 | 5.88 ± 0.97   | 68.3 ± 0.6 | 0.51 ± 0.04   |
| TGM1        | 23.8 ± 0.3 | 5.78 ± 0.08   | 66.0 ± 0.9 | 0.55 ± 0.06   |
| TGM2        | 23.0 ± 0.2 | 5.78 ± 0.07   | 62.0 ± 1.1 | 0.67 ± 0.24   |
| TGM3A       | 23.6 ± 0.2 | 5.09 ± 0.07   | 63.5 ± 0.2 | 0.56 ± 0.02   |
| TGM3G       | 23.3 ± 0.1 | 4.30 ± 0.05   | 63.8 ± 0.1 | 0.30 ± 0.03   |
| TGM4        | 23.2 ± 0.2 | 3.55 ± 0.22   | 64.6 ± 0.8 | 0.37 ± 0.09   |
| TGM5        | 23.7 ± 0.1 | 3.20 ± 0.03   | 65.6 ± 0.2 | 0.20 ± 0.10   |
in terms of the glycerol backbone and three fatty acids. These molecules are good candidates for treating APBD and other conditions through membrane lipid therapy, particularly because they modulate the composition and structure of cell membranes, and because they are more stable than nonhydroxylated triacylglycerols (36). This therapeutic approach involves the regulation of the membrane lipid structure, as has formerly been considered to treat other CNS conditions like Alzheimer’s disease or gliomas (37, 38).

Although TH could compensate the cell energy deficit in APBD patients, the molecular and cellular mechanisms of action of such an effect are not fully understood (7). The present study shows that TH influences the interactions of GBE1 with lipids, and that these interactions enhance GBE1 activity in vitro. However, for future therapeutic considerations, TH is cleaved by a lipoprotein lipase and the resulting heptanoate is used as fuel in the liver, muscle, and nerve tissue (7). This metabolism could reduce the bioavailability of TH and its therapeutic effects in patients. In order to overcome this problem, we designed a more stable hydroxylated TH analog (TGM0), a modification that offers some protection from triacylglycerol metabolism by lipases (36). TGM0 significantly increased GBE1Y329S activity in PBMC cells from APBD patients homozygous for that mutation and TGM5 also enhanced GBE1Y329S activity in vitro and ex vivo (Fig. 8). Moreover, there was a good correlation between GBE1Y329S activity in vitro (liposomes) and in APBD patient’s cells (PBMCs) in the presence or absence of different triacylglycerols (control PC with or without TH, TGM0, TGM1, or TGM5; Fig. 5). Furthermore, the differential effects of the various TGMs on WT and GBE1Y329S binding to membranes indicate that this interaction is based on membrane lipid structure and protein-membrane interactions. As indicated above, the effect of the various TGMs used was associated with regulatory effects on the enzyme’s

Fig. 9. Effect of calcium and anionic phospholipids on GBE1 activity and binding to model lipid membranes. Binding of WT GBE1 (A) (open bars) and GBE1Y329S (C) (solid bars) to PC, PC:PS (3:2, mol:mol) or PC:PIP2 (19:1, mol:mol) membranes in the presence (red) or absence (black) of Ca2+. The data represent the mean ± SEM values of from three to four independent experiments. **P < 0.01, effect of Ca2+; #P < 0.05, effect of PS. Correlation between GBE1 (both WT and GBE1Y329S) activity and membrane binding in the presence of Ca2+ (red) for 30 min (B) or 60 min (D). GBE1 activity and binding were expressed relative to the values for the WT protein in the presence PC (100%, black).
activity. Enhancing GBE1Y329S activity by regulating the structure of the membrane could have a therapeutic effect, as the branching activity might overcome the minimum ramification threshold to maintain glycogen stability. In fact, patients who are heterozygous for GBE1Y329S do not manifest the APBD phenotype, which suggests that, while incomplete, the increase in GBE1Y329S activity shown here for TGM0 and TGM5 may have sufficient therapeutic potential.

Thermal stability is a means to investigate protein structure and ligand-protein interactions (13). The higher T_m determined by DSF for GBE1 (48.9°C) with respect to GBE1Y329S (41.68°C) indicates a lower thermal stability for the latter, consistent with the altered folding indicated previously (1). The presence of PC membranes increases the thermal stability of both WT and GBE1Y329S, and notably, TGM5 induces the largest increase in T_m for both WT and GBE1Y329S (more so for the latter; Fig. 6). This increase in thermal stability produced by PC, and specifically by TGM5, in part explains the stronger activity observed for the WT and GBE1Y329S enzymes in the presence of these lipids. Thermal stabilization enhances the activity of many proteins, some of them related to GBE1 [e.g., α-amylase (39)]. The molecular events behind such effects could be related to protein-membrane interactions that may favor protein docking. In addition, direct TGM protein binding could possibly stabilize the misfolded area around the S329 residue in GBE1, as our DSF data suggest (Fig. 7). These results suggest that the thermal stability of purified GBE1Y329S in the presence of PC vesicles could be used as a criterion for APBD drug discovery (13). It is noteworthy that both TGM0 and TGM5 enhanced GBE1Y329S activity,

**Fig. 10.** WT and GBE1Y329S structure. A: Kyte-Doolittle hydrophobicity plots of WT and GBE1Y329S showing changes between the proteins. B: Surface probability plots showing the relevant changes between the WT and mutant proteins. The boxes, red lines, and asterisks show the region around Y/S329 in detail. See Table 3 for hydrophobicity and surface probability values (19 amino acid windows). C: GBE1 structure showing the Y329 amino acid (orange) and the C terminus (yellow).
with cAMP, Ca2+ can activate glycogen phosphorylase in cogen metabolism and energy expenditure and, along hydrophobic regions of the membrane, thereby regulating hydrophobic areas of a protein to interact with inner hydrophobic areas around APBD. If the regulation of multiple key enzymes by Ca2+ is very dynamic, new questions arise, such as the possible role of Ca2+ in the regulation of glycogen debranching enzymes, the involvement of very low GBE1/GYS activity ratios in the origin of polyglucosan bodies or the possible interference of GBE1 in a phase of glycogen degradation when GBE1 should be inactive. Answering these questions may provide important cues for the efficient clearance of polyglucosan bodies in patients with APBD or Lafora disease.

Indeed, significantly and markedly enhanced binding of WT GBE1 to PS-rich membranes is only observed in the presence of Ca2+, and this interaction was associated with markedly weaker GBE1 activity. This response fits the glycogen synthesis/energy storage repressor role of calcium ions, as GBE is required for glycogen synthesis. By contrast, a nonsignificant influence of PS and Ca2+ on both membrane binding and activity was observed for GBE1Y329S, indicating regulatory differences between the WT and mutant enzyme forms: While WT GBE1 is responsive to regulatory cues from Ca2+ and PS, the mutant GBE1Y329S form is not. It will be interesting to test in the future the response of the entire machinery that orchestrates the balance between glycogen synthesis and degradation, GBE, GYS, and glycogen debranching enzyme, to Ca2+ and PS regulation.

There is an inverse correlation between the GBE1/GYS activity ratio and polyglucosan production (28). Thus, considering a similar model that involves GYS, glycogen phosphorylase, GBE1, and glycogen debranching enzymes may help to better understand the origin and development of polyglucosan bodies. Including laforin phosphatase in the equation (43, 44) could also be of interest, as it is thought to participate directly in polyglucosan body formation in Lafora disease, although the same role is still to be shown in APBD. If the regulation of multiple key enzymes by Ca2+ is very dynamic, new questions arise, such as the possible role of Ca2+ in the regulation of glycogen debranching enzymes, the involvement of very low GBE1/GYS activity ratios in the origin of polyglucosan bodies or the possible interference of GBE1 in a phase of glycogen degradation when GBE1 should be inactive. Answering these questions may provide important cues for the efficient clearance of polyglucosan bodies in patients with APBD or Lafora disease. Finally, all these results further highlight the dynamism and the important regulatory role of cell membranes in the context of cell signaling and metabolism, and more specifically, of membrane lipids and the structures they form.

In summary, the results from this work shed light on previously unknown regulatory effects of lipids and Ca2+ on GBE1 and GBE1Y329S. These data will contribute to our understanding of the molecular aspects of glycogen branching in cells and of APBD pathophysiology. In this context, two molecules have been shown to have significant therapeutic potential: the TH analog, TGM0, that could undergo further development as a pharmaceutical drug; and the EPA-derived triacylglycerol, TGM5, which can be developed as a food supplement and has recently been marketed in the European Union (EPApother, Bega Pharmaceuticals)  En
The authors thank Dr. Hasan O. Akman (Columbia University, NY) for the pOTB7 expression vector (1.8 kb) containing the cDNA encoding the human GBE1 protein and Dr. Frank Niesen (Structural Genomics Consortium, Oxford, UK) for his valuable contribution providing the analytic tool, DSF Analysis 3.0. We are grateful to Dr. Josep Pablo Cánaves, Dr. Joan Cifre Bautá, Mª Trinidad García, and Dr. Jeroni Gallmes (University of the Balearic Islands) for their valuable technical support. We also thank BEGA Pharmaceuticals for kindly donating the TGMs.

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