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Malin decreases glycogen accumulation by promoting the degradation of protein targeting to glycogen (PTG)

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Abstract

Lafora disease (LD) is an autosomal recessive neurodegenerative disease that results in progressive myoclonus epilepsy and death. LD is caused by mutations in either the E3 ubiquitin ligase malin or the dual-specificity phosphatase laforin. A hallmark of LD is the accumulation of insoluble glycogen in the cytoplasm of cells from most tissues. Glycogen metabolism is regulated by phosphorylation of key metabolic enzymes. One regulator of this phosphorylation is protein targeting to glycogen (PTG/R5), a scaffold protein that binds both glycogen and many of the enzymes involved in glycogen synthesis, including protein phosphatase 1 (PP1), glycogen synthase, phosphorylase, and laforin. Overexpression of PTG markedly increases glycogen accumulation, and decreased PTG expression decreases glycogen stores. To investigate if malin and laforin play a role in glycogen metabolism, we overexpressed PTG, malin, and laforin in tissue culture cells. We found that expression of malin or laforin decreased PTG-stimulated glycogen accumulation by 25%, and co-expression of malin and laforin abolished PTG-stimulated glycogen accumulation. Consistent with this result, we found that malin ubiquitinates PTG in a laforin-dependent manner, both *in vivo* and *in vitro*, and targets PTG for proteasome-dependent degradation. These results suggest an additional mechanism, involving laforin and malin, in regulating glycogen metabolism.

Lafora disease (LD; OMIM #254780) is an autosomal recessive disease resulting in severe neurodegeneration, epilepsy, and death (1,2). It is one of five major progressive myoclonus epilepsies (PMEs) and is characterized by myoclonus, tonic seizures, and progressive neurological deterioration (3–6). LD patients typically die within ten years of the first seizure, due to complications related to nervous system degeneration (e.g. status epilepticus and aspiration pneumonia) (2). LD is unique among the PMEs because of the patient's rapid neurological deterioration and the accumulation of cytoplasmic non-proteinaceous inclusion bodies called Lafora bodies (LBs).

LBs develop in the cytoplasm of cells from multiple tissues, including brain, kidney, skin, liver, and cardiac and skeletal muscle (1,7–9). While animals normally store carbohydrates as soluble

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Note added in proof: While this manuscript was in review two papers were published with similar findings using different systems. Vilchez et al. demonstrated in N2a neuronal cells that malin and laforin cooperatively decrease the protein levels of PTG and glycogen synthase, and thus inhibit glycogen stores in neurons (64). In addition, Solaz-Fuster et al. demonstrated that malin in concert with laforin decrease PTG levels and glycogen stores in FTO2B hepatoma cells, but they do not affect glycogen synthase levels (65). While both reports elegantly demonstrated the role of malin and laforin in glycogen metabolism and Lafora disease, neither report investigated an endogenous interaction between PTG, and laforin, nor did they recapitulate the ubiquitination of PTG by malin in the presence of laforin *in vitro*. Thus, these two reports together with the work presented here describe a detailed mechanistic explanation of Lafora disease and fully establish the role of malin and laforin in glycogen metabolism.

glycogen, LBs are insoluble accumulations of a “foreign” carbohydrate. Like glycogen, LBs are composed of α -1,4-glycosidic linkages between glucose residues with α -1,6 branches. However, unlike glycogen the branching in LBs is not as ordered and branches occur far less frequently, every 15–30 glucose monomers versus every 12–14 for glycogen (10,11). Another naturally occurring carbohydrate that is similar to LBs is plant amylopectin, the major component of plant starch. Amylopectin is also composed of α -1,4-glycosidic linkages between glucose residues with α -1,6 branches occurring every 12–20 residues. Accordingly, multiple studies have found that the biochemical properties of LBs resemble amylopectin (10–12).

Recessive mutations in the *EPM2A* (epilepsy myoclonus gene 2A) locus are responsible for approximately 48% of LD cases (13–16). *EPM2A* encodes a bi-modular protein, called laforin, that contains a carbohydrate binding module type 20 (CBM 20) at the amino terminus and a dual specificity phosphatase (DSP) domain at the carboxy terminus (16–18). Laforin is conserved in all vertebrates and a subclass of protists (19). Intriguingly, out of the 128 human phosphatases laforin is the only phosphatase that possesses a CBM of any type. CBMs typically target a protein to a carbohydrate and the enzymatic portion of the protein modifies the carbohydrate in some manner (e.g. α -amylase). Consistent with this precedence, we demonstrated that laforin and a laforin-like protein from plants, called SEX4 (20), release phosphate from complex carbohydrates (19,21). Alternatively, GSK3 β is also reported to be a substrate of laforin (22–24), but we and others have been unable to observe this result (21, 25). Therefore, we purposed that laforin and SEX4 are carbohydrate phosphatases (19,21).

Approximately 40% of LD patients have recessive mutations in *EPM2B* (epilepsy myoclonus gene 2B), which encodes the protein malin (13). Malin is also a bi-modular protein, containing a RING domain followed by six NHL domains. Two labs have shown that the RING domain of malin functions as an E3 ubiquitin ligase (23,26) and the NHL domains are predicted to form a β -propeller type protein interaction domain (27,28). Additionally, we found that malin directly binds and ubiquitinates laforin, and targets laforin for degradation (26). Malin was recently shown to ubiquitinate glycogen debranching enzyme (AGL/GDE) and target it for degradation as well (29).

Herein, we demonstrate that malin also ubiquitinates protein targeting to glycogen (PTG/R5), a regulatory subunit of protein phosphatase 1 (PP1). This ubiquitination both targets PTG for degradation and inhibits glycogen accumulation. Additionally, PTG ubiquitination, both *in vivo* and *in vitro*, is dependent on the presence of laforin. Despite the ubiquitination of PTG by malin, we were unable to detect a direct interaction between malin and PTG and postulate that laforin acts as a scaffold protein to facilitate PTG ubiquitination by malin. These results suggest that malin degrades multiple enzymes involved in glycogen metabolism in order to tightly control this process. Thus, when malin is defective glycogen metabolism proceeds aberrantly and LBs are produced.

Experimental Procedures

Plasmids and Proteins

Laforin and malin constructs and protein purification were described previously (18,19,21, 26). PTG family members were amplified from expressed sequence tag clones and inserted into the pcDNA3.1 FLAG (30) and pcDNA3.1-V5 (Invitrogen) eukaryotic expression vectors, while malin was inserted into pcDNA3.1/*myc*-His and into the pcDNA3.1 FLAG as previously described (26). pcDNA3.1 MDM2 and pcDNA3.1 PIRH2 were a gift from Dr. Michael Karin.

Cell Culture and Transfection

Chinese hamster ovary (CHO) cells stably transformed with the insulin receptor, (CHO-IR), were maintained at 37°C with 5% CO₂ in Earle's Minimal Essential Media (Invitrogen) containing 10% FBS, 50 units/ml penicillin/streptomycin, and 50 mg/ml Geneticin (Invitrogen). Subconfluent cultures of CHO-IR cells were transfected with FuGENE (Roche Applied Sciences) according to the manufacturer's protocol. Each transfection was performed with 10 µg of total DNA, and supplemented with pcDNA3.1 when necessary. Transfected cells were allowed to recover 24h prior to harvest for protein expression and 48h prior to harvest for glycogen measurements.

Immunoprecipitations (IPs)

Cell lysates were prepared as described (21). The supernatants were mixed with anti-FLAG M2 affinity gel (Sigma) or anti-myc agarose (Sigma) for 2–4 h at 4°C with constant agitation. The resins were pelleted by centrifugation at 500 × g for 1 min and washed three times with 1 ml lysis buffer. The beads were resuspended in 30 µl of 4X NuPage sample buffer (Invitrogen) and subjected to Western analyses. Denaturing IPs were performed as described (29). Western blots were probed with the following antibodies: α-FLAG (Sigma), α-myc 9E10 (Sigma), α-V5 (Invitrogen), α-ubiquitin (Covance), avidin-HRP (Boston Biochem), α-laforin, α-malin, or α-PTG. The α-laforin, α-malin, and α-PTG antibodies were generated by immunizing rabbits (Cocalico) with recombinant laforin, a fusion protein of GST and the RING domain of malin, or PTG, respectively, and antibodies were affinity purified from the serum with a HiTrap NHS-activated HP affinity column (GE Healthcare) and/or IgG purified with a Montage™ Antibody Purification kit (Millipore). Goat α-mouse-HRP or donkey α-rabbit-HRP (GE Healthcare) were used as needed. The HRP signal was detected using SuperSignal West Pico (Pierce).

Glycogen Measurements

Glycogen measurements were performed as previously described with the following modifications (31). CHO-IR cells were grown in 100 mM dishes and transfected with the appropriate expression construct(s). Each transfection was performed with 10 µg of total DNA, and supplemented with pcDNA3.1 when necessary. All experimental points were done in triplicate or quadruplicate. Cells were washed three times with PBS, frozen at –80° C for 30 minutes, and lysed by three alternate cycles of freezing (dry ice) and thawing (37° C). Cells were harvested in 400 µl of 0.2 M sodium acetate pH 4.8. Glycogen was hydrolyzed to glucose with 500 µl of 250 milliunits/ml amyloglucosidase (Sigma) and incubated at 37° C for 2–8 h with constant rocking. Samples were neutralized with 125 µl of 0.5M NaOH and cleared by centrifugation for 5 minutes at 3000 rpm. Glucose concentrations were determined using the Roche Molecular Biochemicals D-glucose determination kit according to the manufacturer's instructions. 50–200 µl of cell lysate was added to a hexokinase/glucose-6-phosphate dehydrogenase reaction, and the resulting NADPH was measured at 340 nm.

In Vitro Ubiquitination Assay

In vitro ubiquitin assays were performed as previously described (26) with the following changes. PTG was used as a substrate instead of laforin. ³⁵S-labeled-PTG was made *in vitro* using the TNT T7 coupled reticulocyte lysate system (Promega), it was then immunoprecipitated out of the reaction mixture with anti-FLAG M2 affinity gel (Sigma), washed three times with ubiquitin assay buffer, and eluted into 50 µl assay buffer with 10 µg of FLAG peptide (Sigma). 6 µl of immunopurified ³⁵SPTG was used in each reaction. The reaction mixture was incubated at 37°C for 1 hour. Reaction products were separated, transferred, and subjected to Western analysis using avidin-HRP (Boston Biochem) to detect biotinylated-ubiquitin. After detection, blots were washed in TBS/0.1% NaN₃ to abolish the HRP signal, dried, and exposed on film to detect ³⁵S-PTG.

RESULTS and DISCUSSION

Since LD patients develop cytoplasmic accumulations of insoluble carbohydrate, the disease likely involves the mis-regulation of glycogen metabolism. The enzymes regulating glycogen metabolism, as well as detailed mechanisms of how these enzymes are modulated, have been elucidated over the past 40 years (32–35). Glycogen metabolism is subject to multiple levels of regulation all of which impinge on glycogen synthase (GS), the rate-limiting enzyme in synthesis, and/or phosphorylase, the enzyme that catalyzes the phosphorolysis of the α -1,4-glycosidic linkages.

One regulator of these enzymes is PTG (gene *PPP1R3C*), a regulatory subunit of protein phosphatase 1 (PP1). PTG is expressed in all tissues except testis, and is most abundant in skeletal muscle, heart, and liver (36,37). PTG binds both glycogen and the primary enzymes involved in regulating glycogen metabolism, phosphorylase and glycogen synthase (37). Two groups have shown that disruption of the PTG gene product substantially decreases glycogen stores in tissue culture cells and a mouse model (38,39). In addition, PTG directly interacts with laforin (40). For these reasons, we hypothesized that laforin and/or malin might inhibit the activity of PTG, either directly or indirectly.

Co-expression of malin and laforin reduces glycogen accumulation

Overexpression of PTG leads to an increase in glycogen accumulation in tissue culture cells and *ex vivo* organ models (41–44). We sought to test the hypothesis that malin and laforin oppose the action of PTG. In order to gain insights into the mechanistic nature that malin and laforin play in glycogen metabolism, we measured glycogen accumulation in tissue culture cells expressing various combinations of PTG, laforin, and malin.

We utilized CHO-IR cells since they do not express PTG and produce a minimal amount of glycogen under normal tissue culture conditions (Fig. 1A & B) (37). However, they exhibit a seven-fold increase in glycogen production when transfected with PTG (Fig. 1A & B) (37). We transfected CHO-IR cells with a combination of PTG, laforin, and/or malin to determine how laforin and malin affect glycogen stores. Cells transfected with PTG and either laforin or malin yielded a 25% reduction in glycogen amounts compared to cells transfected with PTG and a vector control (Fig. 1A). This reduction was not dependent on the phosphatase activity of laforin, because transfection with PTG and the catalytically inactive laforin^{C266S} yielded a similar reduction in glycogen stores (Fig. 1A). However, the reduction was dependent on the protein-protein interaction domain of malin. Cells transfected with PTG and a LD disease mutation in the NHL domain of malin, malin^{E280K}, contained similar glycogen stores as vector control cells (Fig. 1A). The effect of malin and laforin in concert was quite striking. Cells transfected with PTG, malin, and laforin contained a similar amount of glycogen as cells transfected with a vector control (Fig. 1A). Therefore, malin and laforin each independently decreased PTG-stimulated glycogen accumulation, and cumulatively, they essentially eliminated PTG-stimulated glycogen accumulation.

To determine if this result was a non-specific affect of overexpressing an E3 ubiquitin ligase, we transfected cells with PTG and MDM2, the E3 ligase that negatively regulates p53 (45–47). While cells transfected with wild-type malin decreased PTG-stimulated glycogen stores, MDM2 did not reduce PTG-stimulated glycogen stores (Fig. 1B). In a similar manner, we wished to ensure that simply overexpression of another gene or the addition of another plasmid did not reduce PTG-stimulated glycogen stores. Therefore, we transfected cells with PTG and either DJ-1, a Parkinson's disease gene, or pEB6 (empty vector). In each case the transfected cells accumulated a similar amount of PTG-stimulated glycogen stores as PTG alone (Fig. 1B). Therefore, the decrease in PTG-stimulated glycogen stores was not an artifact generated by a second vector, another gene being expressed, or expression of another E3 ubiquitin ligase.

PTG is one of five regulatory subunits that target PP1 to glycogen and modulate glycogen accumulation (36,37,41–44,48,49). We investigated if malin and laforin specifically inhibit PTG-stimulated glycogen stores, or if they inhibit stimulated glycogen stores of other PTG family members. PTG and R6 (gene *PPP1R3D*) are expressed in a wide range of human tissues, including the brain (36,37). The other three PP1 targeting subunits display tissue specific expression patterns in skeletal muscle, and/or heart and liver tissue (48–50). One such subunit is G_L (gene *PPP1R3B*), which is expressed in muscle and liver tissue (48,50). To test our hypothesis, we transfected cells with PTG, R6, or G_L alone, and along with malin and laforin. As expected, PTG, R6, and G_L all stimulated increased glycogen accumulation (Fig. 1C). Malin and laforin inhibited R6-stimulated glycogen accumulation, but they did not inhibit G_L -stimulated glycogen accumulation (Fig. 1C). Therefore, malin and laforin inhibit PP1 regulatory subunit-stimulated glycogen accumulation of some PP1 regulatory subunits, PTG/R5 and R6, but not all of them. Interestingly these are the only two regulatory subunits that exhibit a wide expression pattern and are expressed in brain tissue (36,37).

PTG is ubiquitinated and targeted for degradation in a laforin- and malin-dependent manner

We previously reported that malin promotes the ubiquitination and degradation of laforin and stated that this result, while correct, is in conflict with our understanding of LD genetics (26). In addition, we postulated that malin likely promotes the degradation of another protein(s) and that this protein(s) may be a regulator of glycogen synthesis (26).

To test if PTG could be a target of malin, we transfected CHO-IR cells with FLAG-tagged PTG along with myc-tagged malin and untagged laforin, immunoprecipitated PTG with α -FLAG beads, and probed for PTG expression with α -FLAG. We observed a slight reduction in PTG protein levels when cells were co-transfected with laforin alone compared to empty vector (Fig. 2A), and consistently a greater reduction in PTG protein levels in cells co-transfected with malin alone (Fig. 2A). When we transfected cells with FLAG-PTG and both malin-myc and laforin we observed a robust decrease in the protein levels of PTG (Fig. 2A). To determine if the laforin-dependent reduction in PTG was dependent on the phosphatase activity of laforin, we transfected cells with FLAG-PTG, malin, and laforin^{C266S}. Cells transfected with laforin^{C266S} displayed similar decreased levels of PTG compared to cells transfected with wild type laforin (Fig. 2A), demonstrating that this reduction was not dependent on the phosphatase activity of laforin. Therefore, malin and laforin in concert significantly reduced PTG protein levels, and this reduction is not dependent on the laforin's phosphatase activity.

To determine if the interaction between malin and laforin was necessary to enhance the reduction in PTG, we transfected cells with FLAG-PTG and a mutant NHL-domain version of malin, malin^{E280K}-myc, which disrupts its interaction with laforin (26). These cells accumulated a similar amount of FLAG-PTG as vector control cells and substantially more than cells transfected with wild-type malin (Fig. 2B). In addition, cells transfected with FLAG-PTG, laforin, and the E3 ubiquitin ligases Pirh2 or MDM2 accumulated similar amounts of FLAG-PTG as cells transfected with no E3 ubiquitin ligase (Fig. 2B). Thus, malin promotes a decrease in the accumulation of PTG, this decrease is dependent on the interaction between malin and laforin, and the decrease is not promoted by other E3 ubiquitin ligases.

Since malin is an E3 ubiquitin ligase (26), it seemed plausible that malin could ubiquitinate PTG and target it for 26S proteasome-dependent degradation. To test if the decrease in PTG was dependent on the 26S proteasome, we performed similar experiments as above but in the presence and absence of the proteasome inhibitor MG132. Cells transfected with wild-type malin-myc and laforin and treated with MG132 accumulated similar PTG protein levels as vector control cells and substantially more than cells not treated with MG132 (Fig. 2C, top panel). As a positive control, we monitored the protein levels of laforin in the presence and

absence of MG132. As we would predict, since malin ubiquitinates laforin, laforin protein levels also increased in cells treated with MG132 (Fig. 2C, bottom panel). Consistent with these results, when we performed a denaturing immunoprecipitation of FLAG-PTG we found that cells co-transfected with malin-myc and laforin and treated with MG132 accumulated a significant amount of ubiquitin attached to PTG (Fig. 2D). In addition, there was a substantial amount of FLAG-PTG that ran as a high molecular weight smear in the presence of MG132, similar to the detected ubiquitin smear (Fig. 2D). However, treatment of cells with MG132 in the absence of transfected malin-myc also resulted in a high molecular weight smear of PTG and ubiquitin (Fig. 2D). Similarly, we observed that transfection with laforin alone slightly decreased the protein levels of PTG (Fig. 1A). This high molecular weight smear and the decrease in PTG in the absence of malin-myc is likely due to endogenous malin, since we detected malin mRNA in CHO-IR cells (data not shown). Cumulatively, these results strongly suggest that PTG is modified by the attachment of multiple ubiquitins in a malin-dependent manner, leading to the high molecular weight smear, and proteasome-dependent degradation.

To determine if PTG serves as a substrate for malin *in vitro*, we performed *in vitro* ubiquitination assays using recombinant, purified GST-malin-His₆ and ³⁵S-labeled *in vitro* translated PTG. Although we attempted this reaction several times under various conditions, including the conditions used for malin ubiquitination of ³⁵S-laforin (26), we did not observe ubiquitination of ³⁵S-PTG (data not shown).

Given the lack of *in vitro* ubiquitination of PTG, we tested if malin and PTG directly interacted using recombinant, purified GST-malin-His₆ and ³⁵S-labeled *in vitro* translated PTG. Despite utilizing multiple conditions, we did not observe a direct interaction between malin and PTG (data not shown). In absence of evidence of a direct interaction, we asked if malin and PTG co-immunoprecipitate. We transfected cells with FLAG-malin and PTG-V5, treated cells with DMSO or DMSO + MG132, immunoprecipitated with α -V5, and blotted with α -V5 and α -FLAG. While malin did not coimmunoprecipitate with PTG under standard tissue culture conditions, malin did consistently coimmunoprecipitate with PTG in the presence of MG132 (Fig. 3A). Therefore, while malin and PTG do not directly interact, they are in a complex when the 26S proteasome is inhibited.

Interestingly, PTG directly interacts with both laforin and malin *via in vitro* binding experiments, and they interact in both yeast two-hybrid assays and overexpression studies in tissue culture cells (26,40). While PTG interacts with laforin *via* three exogenous assays, there have been no reports that endogenous PTG and laforin co-immunoprecipitate. Therefore, we tested if endogenous PTG and laforin interact. We immunoprecipitated PTG and blotted for PTG and laforin (Fig. 3B). Endogenous PTG and laforin readily co-immunoprecipitated from tissue culture cells and this interaction was increased in the presence of the proteasome inhibitor MG132 (Fig. 3B). Therefore, endogenous PTG and laforin are in a complex with each other, a complex likely involving a direct interaction between PTG and laforin. Since laforin and PTG are both degraded by the 26S proteasome, this interaction is increased when the proteasome is inhibited.

Given our glycogen assay results and the fact that PTG and laforin co-immunoprecipitate, it seemed plausible that laforin could act as a scaffold to allow malin to interact with and ubiquitinate PTG. Therefore, we performed the same *in vitro* ubiquitin assay as above, but included recombinant laforin-His₆. Ubiquitination of ³⁵S-PTG was evaluated by Western blot analysis using avidin-HRP to detect biotinubiquitin and autoradiography to monitor the migration of ³⁵S-PTG. Malin ubiquitinated PTG in the presence of laforin, generating a high molecular weight shift in ³⁵S-PTG that co-migrated with high molecular weight ubiquitin (Fig. 3C). If laforin was removed from the reaction, then PTG was not ubiquitinated (Fig. 3C). Similarly, when malin^{E280K}, which does not efficiently bind laforin (26), was utilized PTG

was not ubiquitinated (Fig. 3C). This result demonstrates that laforin acts as a scaffold between malin and PTG to allow malin to ubiquitinate PTG.

Cumulatively, these results demonstrate that malin ubiquitinates PTG and targets it for proteasome-dependent degradation, and this degradation decreases glycogen accumulation. In addition to PTG, malin ubiquitinates laforin and targets it for degradation (26). Malin was also recently shown to ubiquitinate glycogen debranching enzyme (AGL/GDE) and target it for proteasome-dependent degradation (29). The ubiquitination of AGL also occurred in the absence of a detectable interaction between malin and AGL. Similarly, we now demonstrate that malin ubiquitinates PTG in the absence of a detectable direct interaction. Therefore, we propose that laforin acts as a scaffold to tether malin in the vicinity of at least two substrates (and potentially others), AGL and PTG. In the absence of malin or laforin, PTG levels are increased and increased PTG levels cause glycogen synthase to be hyperactive (39,42). Hyperactive glycogen synthase is one component driving the formation of LBs in LD patients. In support of this explanation, overexpression of glycogen synthase in muscle has been shown to produce a LB-like accumulation (51), likely due to an imbalance between glycogen synthase and glycogen branching enzyme. These results suggest a new model when considering Lafora disease (Fig. 4).

As illustrated in Figure 4, we propose that phosphate is incorporated in glycogen, as a by-product of normal glycogen synthesis. There are reports of phosphate being present in mammalian glycogen (52), and phosphorylation of plant starch is a highly coordinated mechanism (53,54). In addition, LBs contain excess phosphate compared to glycogen (10, 55). Once a glycogen particle forms, PTG and AGL are both targeted to the particles *via* their CBMs, and are both involved in glycogen synthesis (37,56). Laforin too is targeted to glycogen *via* its CBM and once attached it dephosphorylates glycogen, an activity novel to laforin and laforin-like phosphatases (19,21). After laforin has bound glycogen, malin directly interacts with laforin and ubiquitinates laforin, AGL, and PTG (26,29). This ubiquitination releases all three proteins from glycogen and both targets them for proteasome-dependent degradation and allows glycogen metabolism to proceed normally. Thus, laforin plays a role in regulating multiple proteins that drive glycogen metabolism. In support of this role for laforin, data from multiple mouse models demonstrate that laforin protein levels closely correlate with the levels of intracellular glycogen (57). While our model does not account for all aspects of glycogen metabolism, it provides a foundation in understanding multiple facets of Lafora disease.

In this model, malin in essence behaves as a uni-directional signal, and laforin acts as both an activator and repressor of proper glycogen metabolism. The CBM of laforin localizes it to glycogen and positions laforin to dephosphorylate glycogen; this dephosphorylation is an “activator” of glycogen metabolism. In the absence of this dephosphorylation, some aspect of glycogen synthesis is “impaired”, possibly branching, and this is one component that leads to a LB. After dephosphorylation, laforin and additional proteins (such as PTG and AGL) must be degraded or they inhibit proper glycogen metabolism. Thus, laforin is analogous to previously described “activation by destruction” transcriptional activators (58–60), where laforin provides a positive “signal” for glycogen metabolism but then must be degraded for glycogen metabolism to continue. If laforin is lacking then glycogen becomes hyperphosphorylated, glycogen debranching enzyme (AGL/GDE) is overactive which results in fewer branches, and glycogen synthase is overactive due to increased phosphatase activity of PP1-PTG and that also leads to fewer branches. These two characteristics, increased phosphorylation and decreased branches, are both hallmarks of LBs.

This model ascribes laforin two functions in suppressing LD, 1) dephosphorylation of glycogen in order to inhibit LB accumulation (19,21), and 2) it recruits malin to the site of glycogen accumulation so that malin can ubiquitinate PTG, AGL, and laforin (26,29). These concerted

events target PTG, AGL, and laforin for proteasome-dependent degradation, thereby inhibiting LB accumulation and allowing glycogen metabolism to proceed normally. The two roles for laforin and one for malin in inhibiting LD are consistent with patient data which demonstrate that mutations in laforin results in a more severe phenotype and shorter patient life-span than mutations in malin (61–63). Thus, the more biochemical interactions and mechanisms we uncover concerning malin and laforin, the more we understand this complex, neurodegenerative disease.

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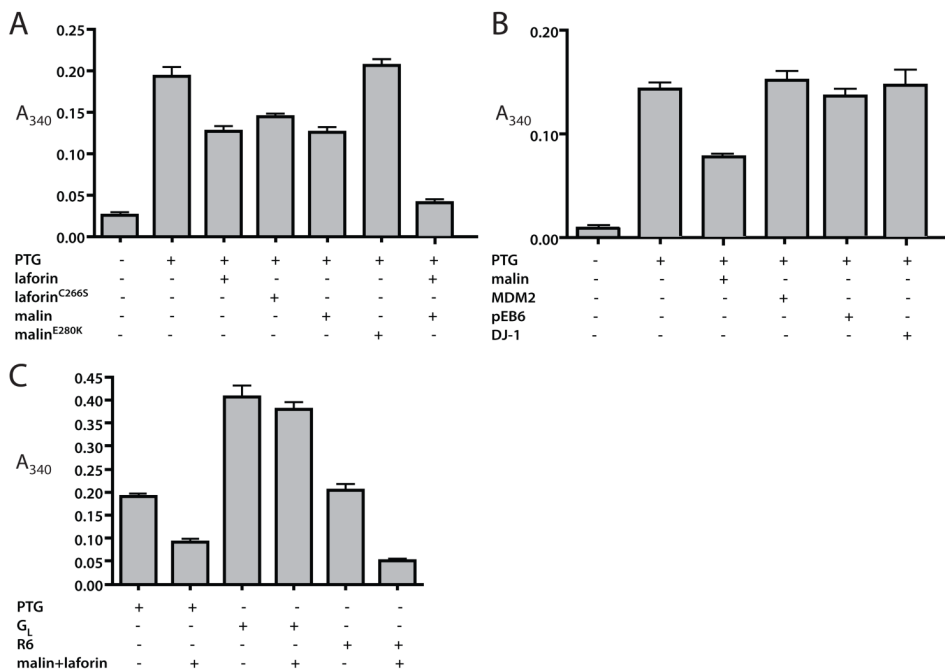


Figure 1. Malin and laforin inhibit PTG/R5- and R6-stimulated glycogen accumulation
A, Eukaryotic expression constructs for PTG, wild type laforin, catalytically inactive laforin (C266S), wild type malin, or a malin NHL-domain mutant (E280K) were transfected into CHO-IR cells as indicated. Cellular glycogen levels were measured 48 h post transfection as described in Experimental Procedures. *B*, Eukaryotic expression constructs for PTG, malin, MDM2, DJ-1, or pEB6 (an empty vector) were transfected into CHO-IR cells as indicated. Cellular glycogen levels were measured as described above. *C*, Eukaryotic expression constructs for PTG, GL, and R6 either with or without malin and laforin were expressed in CHO-IR cells. Cellular glycogen levels were measured as described in *Experimental Procedures*. In each case, results are the mean of triplicate or quadruplicate experiments. In addition, the appropriate amount of pCDNA3.1 was included to bring the total amount of transfected DNA to 10 μ g. Glycogen content is represented by the conversion of glucose (produced by the action of amyloglycosidase on glycogen) to glucose-6-phosphate concomitant with the production of NADPH that was measured *via* a spectrophotometer at 340_{NM}.

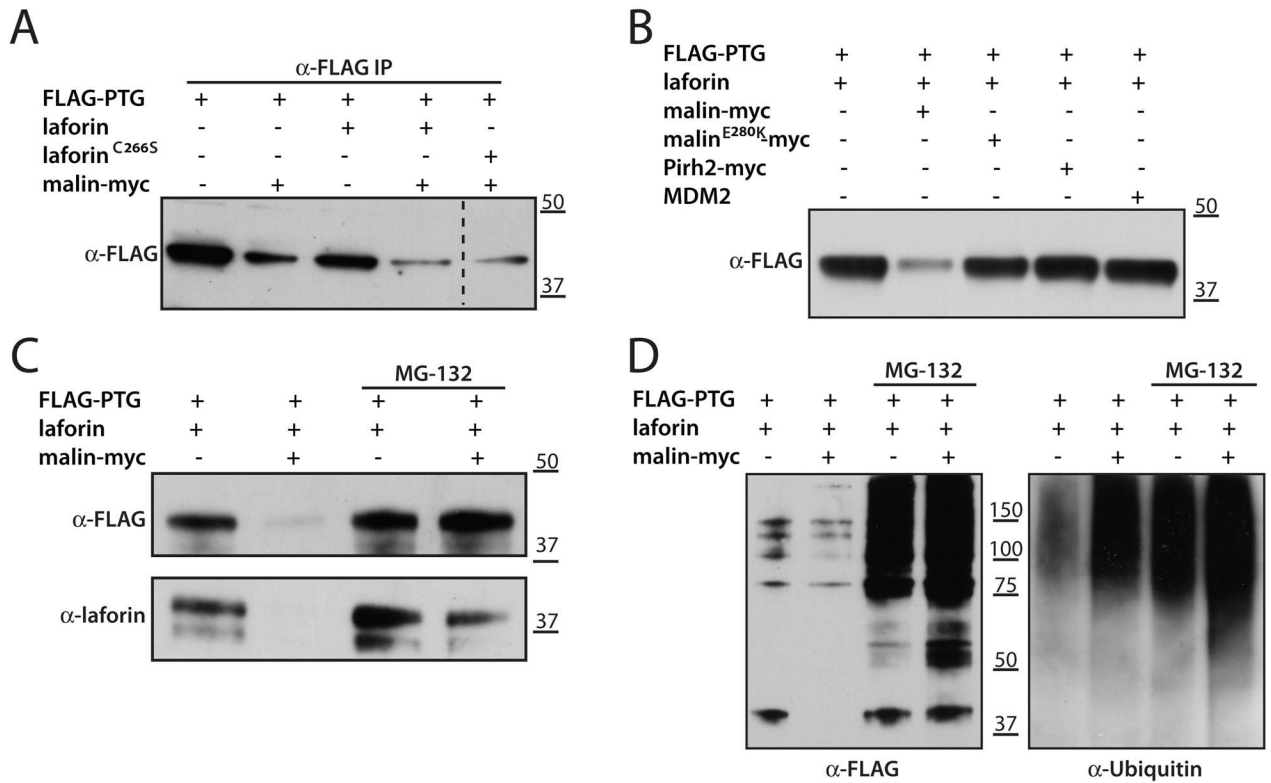


Figure 2. PTG protein levels are decreased in a malin-, laforin-, and proteasome-dependent manner

A, B, CHO-IR cells were transfected with the vectors indicated, lysates were equalized for total protein, immunoprecipitated (IP) with mouse α -FLAG agarose, and immunoblotted with rabbit α -FLAG. In panel *A*, one lane was removed from this blot. IP, immunoprecipitation. *C*, CHO-IR cells were transfected with the vectors indicated, treated with DMSO or DMSO + 10 μ M MG132 for 8 hours, cell lysates were equalized for total protein, immunoprecipitated with mouse α -FLAG agarose, and immunoblotted with rabbit α -FLAG. In addition, whole cell lysate was immunoblotted with rabbit α -laforin. *D*, CHO-IR cells were transfected with the vectors indicated, and treated with DMSO or DMSO + 10 μ M MG132 for 8 hours. Lysates were prepared in denaturing IP buffer, boiled for 10 minutes, diluted with standard IP buffer, equalized for total protein, immunoprecipitated with mouse α -FLAG agarose, and immunoblotted with rabbit α -FLAG and mouse α -ubiquitin.

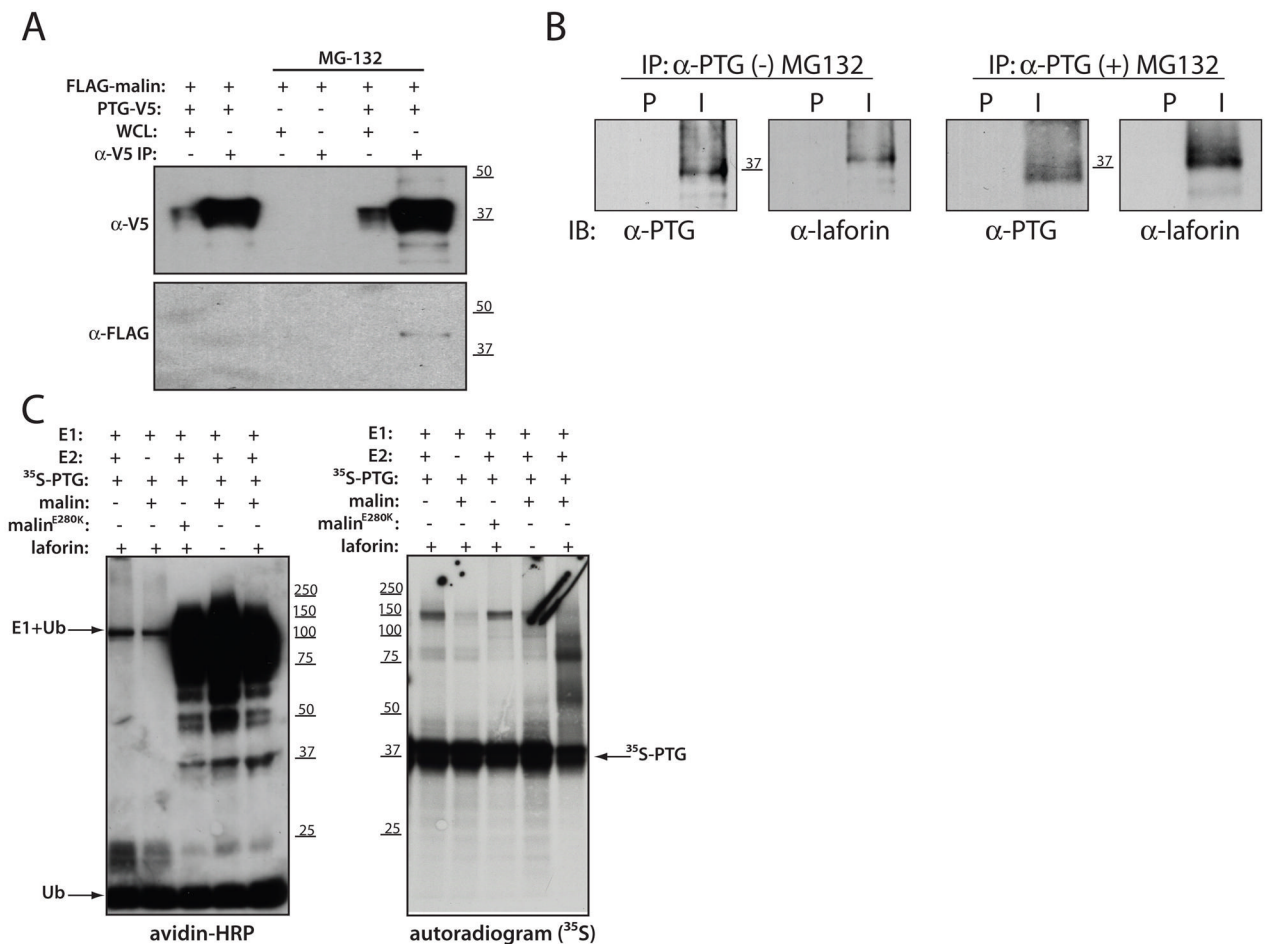


Figure 3. Malin ubiquitinates PTG

A, CHO-IR cells were transfected with the vectors indicated, treated with DMSO or DMSO + 10 μM MG132 for 6 hours, cells were lysed, lysates were equalized for total protein, immunoprecipitated (IP) with mouse α -V5 agarose, and immunoblotted with mouse α -V5-HRP and mouse α -FLAG-HRP. Additionally, whole cell lysate (WCL) was immunoblotted with both mouse α -V5-HRP and mouse α -FLAG-HRP. **B**, HEK293 cells were treated with DMSO or DMSO + 10 μM MG132 for 6 hours, cells were lysed, lysates were equalized for total protein, immunoprecipitated (IP) with pre-immune serum (P) or affinity purified α -PTG immune (I), and immunoblotted (IB) with both α -laforin and α -PTG. Immunoblots from IPs (-) MG132 were exposed for 90–120 seconds and blots from IPs (+) MG132 were exposed for 15–30 seconds. **C**, *In vitro* ubiquitin assays were performed as described in *Experimental Procedures* with E1 enzyme, E2 (UbcH5a), ATP, biotinylated ubiquitin, untagged ubiquitin, ^{35}S -labeled PTG (^{35}S -PTG), plus or minus recombinant laforin, and either recombinant GST-malin-HIS₆ or recombinant GST-malin-HIS₆^{E280K}. Reactions were immunoblotted with avidin-HRP to detect ubiquitination, the peroxidase reaction was quenched with 0.1% NaN₃, and the migration of PTG was detected *via* autoradiography of ^{35}S .

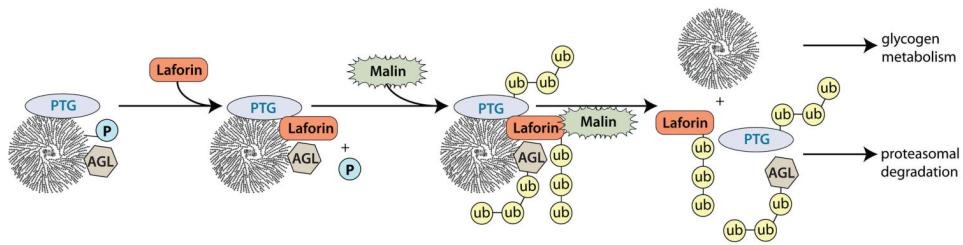


Figure 4. Model of the mechanisms of Lafora disease

PTG (protein targeting to glycogen) and AGL (glycogen debranching enzyme) both bind nascent glycogen particles *via* their carbohydrate binding modules (CBM), during normal glycogen metabolism. Glycogen is phosphorylated (P) during an early step of glycogen metabolism. Laforin is then targeted to the glycogen particle *via* its CBM and releases the phosphate from glycogen. In addition, laforin directly interacts with PTG. *Via* an unknown signal, malin is targeted to laforin and binds laforin *via* its NHL domain. Once bound to laforin, malin ubiquitinates laforin, PTG, and AGL. This ubiquitination triggers the release of all of these enzymes from the glycogen particle and both targets them for proteasome-dependent degradation and allows glycogen metabolism to proceed normally.