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Relationship between glycogen accumulation and the laforin dual specificity phosphatase

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Abstract

Laforin, encoded by the *EPM2A* gene, is a dual specificity protein phosphatase that has a functional glycogen binding domain. Mutations in the *EPM2A* gene account for around half of the cases of Lafora disease, an autosomal recessive neurodegenerative disorder, characterized by progressive myoclonus epilepsy. The hallmark of the disease is the presence of Lafora bodies, which contain polyglucosan, a poorly branched form of glycogen, in neurons and other tissues. We examined the level of laforin protein in several mouse models in which muscle glycogen accumulation has been altered genetically. Mice with elevated muscle glycogen have increased laforin as judged by Western analysis. Mice completely lacking muscle glycogen or with 10% normal muscle glycogen had reduced laforin. Mice defective in the *GAA* gene encoding lysosomal α-glucosidase (acid maltase) overaccumulate glycogen in the lysosome but did not have elevated laforin. We propose, therefore, that laforin senses cytosolic glycogen accumulation which in turn determines the level of laforin protein.

Lafora disease is an autosomal recessive, progressive myoclonus epilepsy (OMIM #254780), with onset typically in the teenage years followed by progressive decline and death usually within ten years [1–3]. A hallmark of the disease is the presence of Lafora bodies which are periodic acid-Schiff (PAS) positive structures containing polyglucosan, an abnormal form of glycogen that is poorly branched. Although the pathology is thought to be due to the presence of Lafora bodies in neurons, they are present in other tissues including liver, muscle and skin [4–7]. Mutations in two genes, *EPM2A* and *EPM2B*, are about equally common and together account for approximately 90% of Lafora cases [1,3]. *EPM2A*, which has been more studied, encodes a dual specificity protein phosphatase called laforin [8] that contains a functional polysaccharide binding domain [9–11]. Laforin binds preferentially to polyglucosan structures over glycogen [12], an observation consistent with the fact that recombinant laforin is much more sensitive to inhibition by amylose or starch than glycogen [13]. Approximately forty mutations have been identified to date and these are distributed throughout all four exons of the *EPM2A* gene (http://projects.tcag.ca/lafora/)[8,11,14–17]. When transferred to recombinant laforin produced in *Escherischia coli*, most mutations cause a loss of phosphatase

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activity [10,18]. However, one disease mutation, W32G, in the polysaccharide binding domain eliminates glycogen binding [9,10,13] without abolishing phosphatase activity [9,13]. Therefore, binding to polysaccharide is required for normal laforin function *in vivo*.

The *EPM2B* gene (also called NHLRC1) encodes malin, a 395 residue protein that contains an NH_2-terminal RING finger domain followed by six NHL domains [19]. The RING finger domain is characteristic of E3 ubiquitin ligases [20] and Gentry et al. [21] reported that malin interacts with laforin and catalyzes its polyubiquitination *in vitro* and in cultured cells. In cells, the result is degradation of laforin. However, it is hard to reconcile the destruction of laforin by malin with the fact that Lafora disease is caused by recessive mutations in either *EPM2A* or *EPM2B*. On the model of Gentry et al. [21], defective malin should up-regulate laforin. Lohi et al. [22] found that malin interacts also with glycogen synthase and propose that the laforin-malin-glycogen synthase complex is targeted for degradation. In the same study, it was also reported that laforin could dephosphorylate the NH_2-terminal inhibitory phosphorylation site of the protein kinase GSK-3 although others have challenged this idea [23]. A recent paper has suggested that laforin is a carbohydrate phosphatase as it was reported to release phosphate from amylopectin [23].

Despite these significant advances, the cause of Lafora disease and the reason for the accumulation of Lafora bodies is not understood. In further efforts to link glycogen metabolism with laforin, we took advantage of several genetically modified mouse lines in which glycogen stores are increased or decreased. We observed that the level of laforin protein varies in proportion to glycogen accumulation in the cytosol, providing further evidence for a genetic connection between glycogen and laforin.

Materials and methods

Genetically modified mouse models

GSL30 mice express rabbit muscle glycogen synthase in which two critical inactivating phosphorylation sites, 2 and 3a, are mutated to Ala [24]. Expression is under the control of the muscle creatine kinase promoter and the animals overaccumulate glycogen in skeletal muscles. Lexicon Genetics Incorporated, The Woodlands, TX, utilizing their OmniBank™ library of gene-trapped mouse embryonic stem (ES) cell clones, generated *Gys1* heterozygous null mice. Crossing of these heterozygous mice gave us homozygous null mice (MGSKO) whose muscles are devoid of glycogen [25]. *Gys1* is one of two glycogen synthase genes, and is expressed in most tissues other than liver. Mice with the type 1 phosphatase glycogen-binding subunit R_{GL} (also known as G_M) disrupted accumulate only 10% as much muscle glycogen as wild-type animals [26]. Mice that over-express R_{GL}under control of the muscle creatine kinase promoter have 3–4 times the wild-type muscle glycogen content [27]. GAA −/− mice are a model of Pompe disease in which the gene encoding the lysosomal α-glucosidase (acid maltase) is disprupted [28].

Preparation of mouse tissues

Mouse tissues were harvested as rapidly as possible and frozen immediately in liquid nitrogen. Tissues were later powdered by a tissue pulverizer and stored at −80ºC. Samples of frozen tissues were homogenized with a Tissue Tearer (Biospec Products Inc.) in 10 or 30 volumes of a buffer containing 50 mM Tris-HCl pH 7.8, 2 mM EGTA, 10 mM EDTA, 100 mM NaF, 0.5 mM PMSF, 2 mM benzamidine, 5 0 mM β–mercaptoethanol, 10 mg/ml leupeptin and 37 μg/ml TLCK. Protein concentration was measured by the method of Bradford [29] using bovine serum albumin as standard.

Generation of anti-laforin antibody

Recombinant mouse laforin was produced in *E. coli* and purified as previously described [13]. The anti-laforin antibody was raised in rabbits using recombinant laforin protein as antigen by Cocalico Biologicals, Inc. The antibodies were affinity purified using recombinant laforin coupled with Affigel-15 (Bio-Rad). After dialysis against 100 mM MOPS pH 7.5 overnight at 4ºC, laforin (5 mg) was incubated with Affigel-15 overnight at 4ºC. Then 0.1 M ethanolamine-HCl pH 8 was added to block any unreacted sites. The resin was packed into a column and washed with PBS (0.27 mM KCl, 0.15 mM KH_2PO_4, 14 mM NaCl and 0.81 mM Na_2HPO_4, pH 7.5), 100 mM MOPS pH 7.5 and 100 mM glycine HCl pH 2.4/150 mM NaCl. Serum was passed through the column three times and the column was washed with PBS. Affinity purified antibody was eluted with 100 mM glycine HCl pH 2.4, 150 mM NaCl and immediately neutralized with Tris HCl pH 8.0. The antibody was dialyzed against PBS overnight, glycerol was added to 25% (v/v) and the antibody was stored at −80ºC.

Western Blotting

Four volumes of SDS loading buffer was added to one volume of whole homogenate and boiled. Loading buffer was 62.5 mM Tris-HCl, pH 6.8, 50% (v/v) glycerol, 2.5% (w/v) SDS, 5% (v/v) β-mercaptoethanol, and 1.25% (w/v) bromophenol blue. Typically 30 μg protein was loaded per lane and separated by SDS-PAGE with 10% acrylamide. The protein was transferred to nitrocellulose and incubated with the indicated first antibody. Horseradish peroxidase-conjugated secondary antibody (Sigma) and Enhanced Chemiluminescence (Amersham) were used for detection. Autoradiograms were quantitated by densitometric scanning of films by using Kodak 1D Image Analysis Software (Kodak). Results are presented as means ± standard error of the mean. Unpaired Student's t-test was used to determine statistical significance using the Statview package (Abacus Concept, Inc.). A value of $p<0.05$ was considered significant.

Measurement of glycogen

Glycogen content in tissue was determined in samples of frozen tissue (~30 mg) by measuring amyloglucosidase-released glucose from glycogen by the method of Bergmeyer [30] as previously described by Suzuki *et al*. [26].

Results and discussion

Since we had at our disposal several relevant mouse models, we asked whether genetic manipulation of muscle glycogen content influenced laforin levels in that tissue. MGSKO mice have the *Gsy1* gene disrupted, resulting in animals whose skeletal muscle is completely devoid of glycogen [25]. The level of muscle laforin is reduced by approximately 60% in these mice (Fig. 1B) with no difference in transcript level detectable by real-time PCR (data not shown). GSL30 mice, which over-express glycogen synthase and have massive over-accumulation of muscle glycogen with Lafora-like bodies, have a 7-fold elevation of muscle laforin content (Fig. 1A). R_{GL} (also called G_M) is a type 1 protein Ser/Thr phosphatase targeting subunit that binds glycogen [31]. Muscle from mice with the corresponding gene disrupted accumulate only 10% of normal muscle glycogen [26] and have 20% the wild-type laforin level (Fig. 1D). In contrast, R_{GL} over-expressors that accumulate 3–4 times the wild-type glycogen in their muscle, have a four-fold increase in laforin (Fig. 1C). There is a good correlation, therefore, between the muscle glycogen content, which is cytosolic, and the level of laforin protein (Fig. 2). GAA −/− mice [28] are a model for Pompe disease in which the gene encoding the lysosomal α-glucosidase is disrupted and the mice accumulate glycogen in their lysosomes. In this case, the overaccumulation of glycogen is associated with a three-fold decrease in laforin level (Fig. 3).

The most important conclusion of this work is that altered glycogen stores correlated positively with laforin protein level in all the mouse models but one, providing further evidence for a genetic link between cytosolic glycogen and laforin. At least in the MGSKO mice, the reduced laforin was not associated with a change in transcript level. An obvious thought is that the presence of glycogen, to which laforin is known to bind, stabilizes the protein and protects it from degradation. If malin is involved in the degradation of laforin, as has been suggested [21], one could envision a mechanism whereby polysaccharide binding in some way reduces the susceptibility of the protein to be ubiquitylated. However, other mechanisms for the stabilization of laforin cannot be excluded.

If laforin has a role in glycogen synthesis and/or disposal, one could further speculate as to whether increased laforin provides feedback on glycogen accumulation. One possibility would be via GSK-3, which has been proposed to be a substrate for laforin [22]. GSK-3 is a protein kinase considered important for the phosphorylation and inactivation of glycogen synthase (see [32] for review) and dephosphorylation of its N-terminal inhibitory phosphorylation site should correspond to inactivation of glycogen synthase. However, whether GSK-3 is a laforin substrate is proving somewhat controversial [23]. The other possible laforin substrate is glycogen itself [23], which has long been known to contain small amounts of covalently attached phosphate [33]. The implications of glycogen dephosphorylation are not yet clear but could have to do with maintaining a sufficiently branched polysaccharide.

The one mouse line which did not fit the correlation between glycogen level and laforin was the GAA −/− line. This model of Pompe disease has massive overaccumulation of glycogen in the lysosomes of skeletal muscle as well as large accumulation of autophagic vesicles containing polysaccharide in some fiber types [34,35]. Our results could be rationalized if laforin did not have access to the bulk of the glycogen present in GAA −/− muscle. The extension of the argument would be that, if laforin does exert some regulatory or monitoring function, it does so by sensing cytosolic glycogen.

Despite a growing understanding of the genetic and biochemical connections among laforin, malin, glycogen and glycogen metabolizing enzymes, the molecular basis for the formation of Lafora bodies remains elusive.

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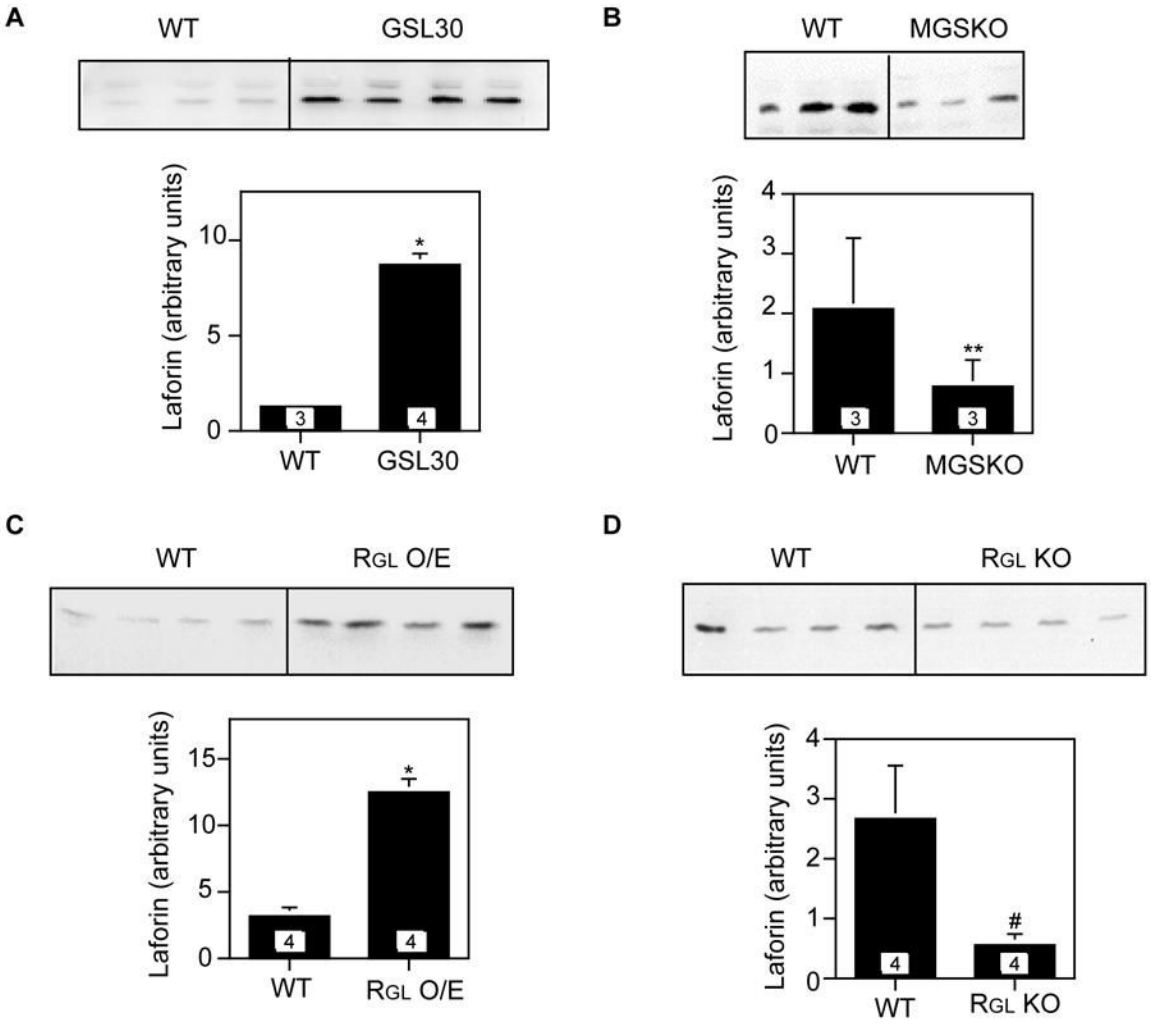

Figure 1.

Laforin expression in skeletal muscle of genetically modified animals with abnormal glycogen levels. The numbers within the columns indicated the mice analyzed. *p<0.0005 vs WT. **p=0.06 vs WT. #p<0.05 vs WT. **A.** Laforin expression in GSL30 mice. **B.** Laforin expression in MGSKO mice. **C.** Laforin expression in R_{GL} overexpressors (R_{GL} O/E). **D.** Laforin expression in R_{GL} knockout mice (R_{GL} KO).

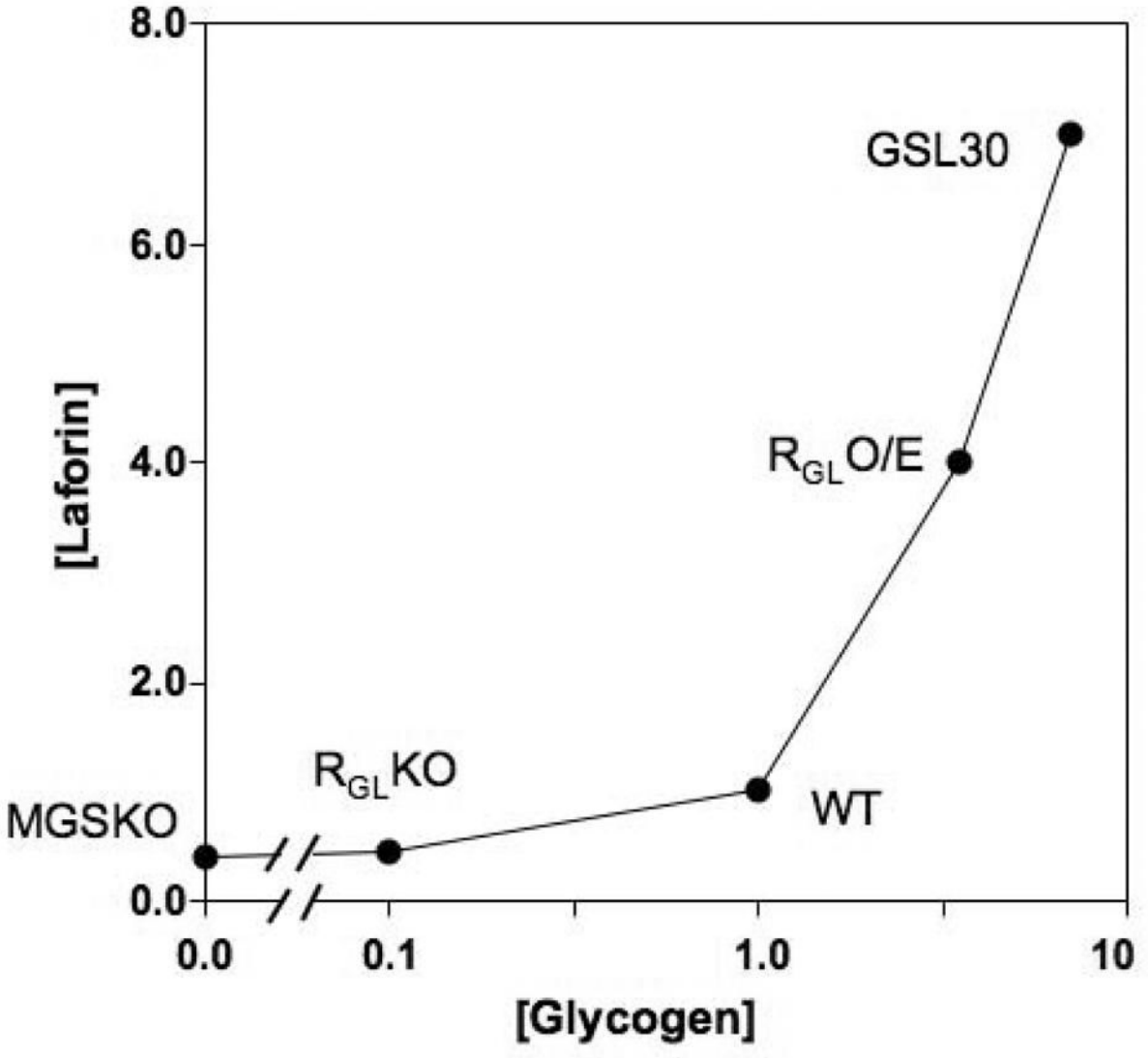

Figure 2.
Correlation between glycogen and laforin levels in muscle. The graph quantitates the data from Fig. 1, setting laforin and glycogen to 1 in the corresponding wild-type controls.

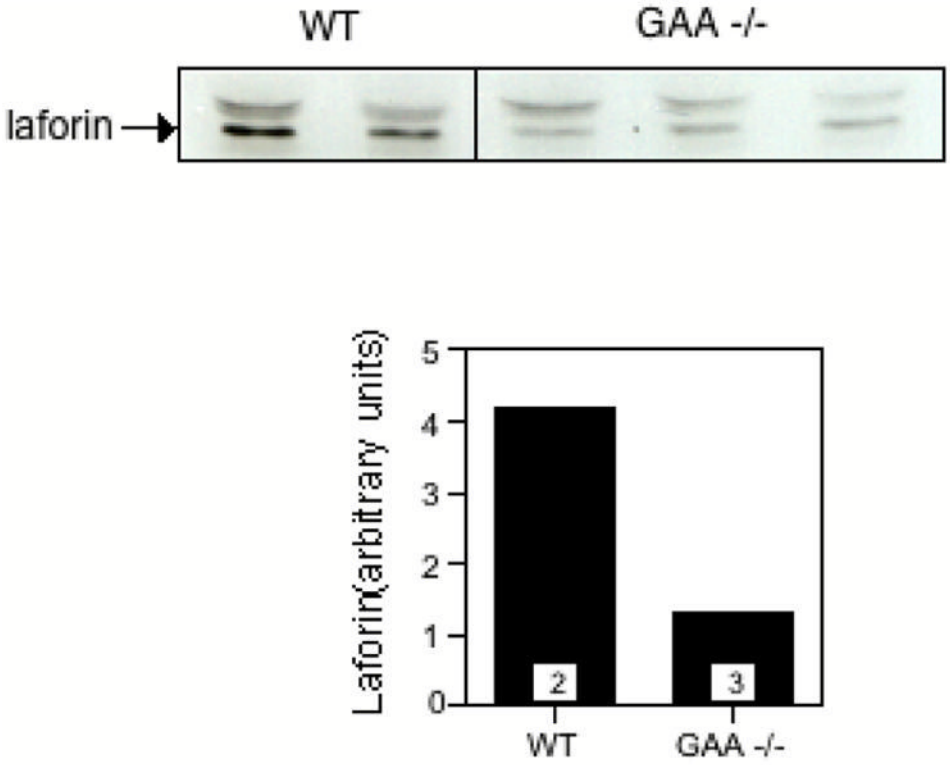

Figure 3.
Laforin expression in GAA −/− mice. The numbers within the bars indicate the number of animals analyzed.