Laforn is the only phosphatase in the animal kingdom that contains a carbohydrate-binding module. Mutations in the gene encoding laforin result in Lafora disease, a fatal autosomal recessive neurodegenerative disorder, which is diagnosed by the presence of intracellular deposits of insoluble complex carbohydrates known as Lafora bodies. We demonstrate that laforin interacts with proteins known to be involved in glycogen metabolism and rule out several of these proteins as potential substrates. Surprisingly, we find that laforin displays robust phosphatase activity against a phosphorylated complex carbohydrate. Furthermore, this activity is unique to laforin, since several other phosphatases are unable to dephosphorylate polysaccharides. Finally, fusing this activity is unique to laforin, since several other phosphatases known to be involved in glycogen metabolism. Normal cells store carbohydrates in the form of glycogen, which is stored in the form of glycogen granules in the cytoplasm of nearly all cell types (2,20–24). Because of this and the fact that laforin contains a CBM, it is hypothesized that laforin is involved in glycogen metabolism, either its synthesis or degradation. Normal cells store carbohydrates in the form of glycogen, a polymer of glucose residues linked together by α-1,4-glycosidic linkages with branches occurring every 8–12 residues via α-1,6-glycosidic linkages (12). Furthermore, this polyubiquitination leads to the degradation of laforin in tissue culture cells (13). Lending support to this surprising finding, Chan et al. (19) reported that although laforin cannot be detected in wild type tissues, it could be detected in EPM2B null tissues. One of the clinical manifestations of LD is the appearance of insoluble carbohydrate deposits called Lafora bodies (LB) in the cytoplasm of nearly all cell types (2,20–24). Because of this and the fact that laforin contains a CBM, it is hypothesized that laforin is involved in glycogen metabolism, either its synthesis or degradation. Normal cells store carbohydrates in the form of glycogen, a polymer of glucose residues linked together by α-1,4-glycosidic linkages with branches occurring every 8–12 residues via α-1,6-glycosidic linkages. This level of branching renders them insoluble (25). Additionally, LBs are composed of the same backbone structure as glycogen, there are fewer α-1,6-glycosidic branches (25). This decreased branching gives LBs a crystalline structure and renders them insoluble (25). Additionally, LBs are significantly
more phosphorylated than glycogen (26). Surprisingly, although LBs and glycogen differ in multiple structural aspects, LBs and amylopectin appear to be very similar.

Amylopectin is the major component of plant starch and is composed of the same backbone structure as glycogen but with branches occurring every 24–30 glucose residues. This decreased amount of branching also renders amylopectin crystalline and insoluble. Additionally, the glucose monomers of amylopectin are phosphorylated on ~1 in every 300 residues at either the C-3 or C-6 position (27). Strikingly, the definitive biochemical studies on the structure of LBs revealed that LBs are more similar to amylopectin than to any other naturally occurring or synthetic compound, including mammalian glycogen (25, 28, 29).

In order to understand the molecular role of laforin in glycogen metabolism, we analyzed its protein-protein interactions in the cell. We further tested interacting proteins for their ability to act as substrates for the phosphatase activity of laforin. Since none of the proteinaceous substrates we tested appeared to be substrates for laforin, we questioned whether laforin could act on a nonproteinaceous substrate. Since LBs are similar to amylopectin, we tested amylopectin as a substrate and demonstrated that laforin effectively removes phosphate from this carbohydrate. We further demonstrate that this activity is specific for the laforin phosphatase and that replacing the laforin phosphatase domain with that of VHR, an active dual specificity phosphatase, does not confer activity toward amylopectin. Finally, we speculate on the consequences this unexpected activity could have on glycogen metabolism.

## MATERIALS AND METHODS

**Plasmids and Proteins**—Wild type and C/S FLAG-tagged laforin for use in mammalian expression studies and bacterially expressed laforin in pET21a (Novagen, San Diego, CA) were described previously (7). PTG family members were amplified from expressed sequence tags and inserted into the pCDNA3.1/myc-His eukaryotic expression vector (Invitrogen). HA-tagged GSK3β was a kind gift from David Pagliarini (Harvard University, Cambridge, MA).

Recombinant His-tagged VHR expressed in *Escherichia coli* BL21 (DE3) CodonPlus RIL cells (Stratagene, La Jolla, CA) was purified using Ni²⁺-agarose (Qiagen, Germany) as described above (30). PTPMT1 was a kind gift from David Pagliarini and Ji Zhou (University of California at San Diego, La Jolla, CA), and dullard was a kind gift from Youngjun Kim (University of California, Cambridge, MA).

**Cell Culture and Transfection**—Adenovirus-transformed human embryonic kidney HEK293T cells were maintained at 37 °C with 5% CO₂ in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum, 50 units/ml penicillin/streptomycin, and 50 μg/ml Geneticin (Invitrogen). Subconfluent cultures of HEK293T or CHO-IR cells (1–2 × 10⁸ cells/100-mm dish) were transfected with FuGENE transfection reagent (Roche Applied Sciences) according to the manufacturer’s protocol. Transfected cells recovered 24–48 h prior to harvest to allow for protein expression.

**Immunoprecipitations (IPs)**—24–48 h after transfection, cells were washed once with ice-cold phosphate-buffered saline, drained, and harvested in ice-cold lysis buffer consisting of 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40 (Nonidet P-40), 1 mM dithiothreitol, and Complete protease inhibitor mixture (Roche Applied Science). The cells were lysed by titration and cleared by centrifugation at 8,000 × g for 10 min. The supernatants were mixed with anti-FLAG M2 affinity resin (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 of lysis buffer. The beads were resuspended in 30 μl of NuPage sample buffer (Invitrogen) and subjected to Western analyses. Western blots were probed with the following antibodies: α-FLAG HRP (Sigma), α-glycogen synthase (α-GS) (Chemicon, Temecula, CA), α-Myc HRP (Roche Applied Sciences), α-HA HRP (Roche Applied Sciences), GSK3β α-Ser(P)⁹ (BiOSOURCE, Camarillo, CA), and α-Tyr(P) 4G10 (Upstate Biotechnology, Inc., Charlottesville, VA). Goat α-mouse HRP was used as needed. The HRP signal was detected by using SuperSignal West Pico (Pierce).

**Isolation of Phosphorylated GSK3β**—CHO-IR cells were transfected with HA-tagged GSK3β and allowed to recover for 24 h. Immediately before harvesting, the cells were treated with 50 nM insulin for 5 min. Extracts were prepared as described above, and α-HA affinity resin (Roche Applied Science) was used to immunoprecipitate GSK3β. The α-HA affinity resin was washed three times with lysis buffer, one time with lysis buffer containing 1 mM NaCl, one time with lysis buffer, and two times with phosphatase buffer. The final product was resuspended in 150 μl of phosphate buffer (1× phosphate reaction buffer: 0.1 M sodium acetate, 0.05 M bis-Tris, 0.05 M Tris-HCl, 2 mM dithiothreitol, pH 6.5), and 20 μl was used in the phosphatase reaction (30 μl total) containing 500 ng of laforin. Tungstate (1 mM) was added prior to the addition of laforin.

**Phosphatase Activity Assays**—Hydrolysis of *para*-nitrophosphophate (pNPP) was performed in 50-μl reactions containing 1× phosphate buffer (above), 50 μM pNPP, and 100–500 ng of enzyme at 37 °C for 1–5 min. The reaction for dullard also contained 10 mM MgCl₂, and the protein phosphatase 1 reaction mix contained 1 mM MnCl₂. The reaction was stopped by the addition of 200 μl of 0.25 N NaOH. Absorbance was measured at 410 nm. Malachite green assays containing 1× phosphate buffer (MgCl₂ or MnCl₂ when appropriate), 100–500 ng of enzyme, and ~45 μg of amylopectin or glycogen were performed in a final volume of 20 μl. Reactions were terminated by the addition of 20 μl of 0.1 M N-ethylmaleimide and 80 μl of malachite green reagent. Absorbance was measured at 620 nm.
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RESULTS AND DISCUSSION

Laforin Is Not a Substrate of Laforin—Laforin is unique among phosphatases found in the animal kingdom in that it contains an NH2-terminal starch-binding domain of the sub-type CBM20 (31). Accordingly, we previously demonstrated that laforin binds to glycogen in vitro (7). In order to elucidate the role of laforin in cellular signaling, we sought to evaluate which proteins involved in glycogen metabolism would co-immunoprecipitate with laforin, with the idea that co-immunoprecipitating proteins could be potential substrates for the phosphatase.

During the course of this study, it was reported that GSK3β co-immunoprecipitated with laforin and that laforin dephosphorylated Ser9 of GSK3β (32). To test these findings, we transfected HA-tagged GSK3β into HEK293 and CHO-IR cells along with FLAG-tagged laforin and immunoprecipitated laforin using anti-FLAG. Despite robust expression of both laforin and GSK3β, GSK3β did not co-immunoprecipitate with laforin from HEK293 or CHO-IR cells (Fig. 1A) (data not shown).

Despite this lack of interaction, we went on to determine if GSK3β was a substrate of laforin. For these experiments, we took advantage of the finding that laforin (C/S) laforin acts as a dominant negative in the mouse model (19), potentially “trapping” the substrate in the phosphorylated form. Thus, we hypothesized that overexpression of C/S laforin in tissue culture cells might “trap” the substrate of laforin in the phosphorylated form. The major regulatory site of phosphorylation on GSK3β is Ser9, and this was the site previously reported to be dephosphorylated by laforin (32). This is a particularly attractive hypothesis to explain the molecular mechanism of LD, since phosphorylation of Ser9 by an upstream kinase, such as Akt, results in inactivation of GSK3β (33, 34). Inactive GSK3β is not able to phosphorylate GS, resulting in a more active form of GS and leading to increased glycogen synthesis. Wild type (WT) or C/S FLAG-tagged laforin along with HA-tagged GSK3β were transiently introduced into HEK293 cells. GSK3β was immunoprecipitated using anti-HA resin, and Western analysis using anti-Ser(P)9 antibody was performed to determine the phosphorylation level of Ser9 in vivo (Fig. 1B). There was no change in the phosphorylation status of this residue upon expression of WT versus C/S laforin. Nonetheless, we pursued the claim that GSK3β is a substrate of laforin and tested whether laforin could dephosphorylate GSK3β in vitro. Cells transiently overexpressing HA-tagged GSK3β were treated with insulin or platelet-derived growth factor to maximally phosphorylate GSK3β on Ser9. GSK3β was immunoprecipitated from cells and subjected to treatment with laforin in the presence or absence of insulin or growth factor. We demonstrated that neither WT GSK3β (top) nor C/S GSK3β (bottom) was a substrate of laforin, as determined by Western analysis using specific antibodies directed against Ser9 of GSK3β (Fig. 1C). Since GSK3β activity is also thought to be regulated by Tyr phosphorylation (35), we tested to see if laforin could dephosphorylate WT GSK3β on Tyr residues using an anti-phosphotyrosine antibody. As shown in Fig. 1D, laforin did not dephosphorylate Tyr residues on GSK3β. In an effort to be fully confident that GSK3β is not a substrate of laforin, we monitored dephosphorylation of GSK3β by radiolabeling cells and checking for changes in the phosphate content of immunoprecipitated GSK3β in the presence of WT versus C/S laforin. These results were also negative (data not shown). Therefore, we conclude that contrary to a published report, GSK3β is not a substrate of laforin.

Laforin Interacts with Proteins Involved in Glycogen Metabolism—In an effort to widen our search for the laforin substrate, we turned our attention to proteins that co-immunoprecipitate with laforin. We previously demonstrated that laforin co-localizes with glycogen synthase (GS) in cells overexpressing both GS and laforin (7). In addition, transgenic mice overexpressing GS in muscle manifest an aberrant form of glycogen that resembles LRs (36). To ascertain if GS co-immunoprecipitates with laforin, WT or catalytically inactive (C/S) FLAG-tagged laforin expression vectors were transfected into CHO-IR cells followed by immunoprecipitation using anti-FLAG. Endogenous GS immunoprecipitated with both WT and C/S laforin (Fig. 2A, left panels). Similarly, both WT and C/S laforin were immunoprecipitated with endogenous GS using antibodies directed against GS (Fig. 2A, right panels). However, efforts utilizing both antibodies directed against phosphorylated GS and radiolabeling of cells overexpressing WT or C/S laforin, followed by analysis of the radioactive labeling of GS, failed to support the hypothesis that GS was a substrate of laforin (data not shown).

Since PTG had previously been shown by two-hybrid analysis to interact with laforin, we next turned our attention to the...
members of the PTG family (12). PTG (R5) and related family members GL, GM, and R6 serve as scaffolds to assemble proteins involved in glycogen metabolism. Although the binding partners of all of the family members have not yet been defined, PTG interacts with enzymes that regulate glycogen metabolism, including protein phosphatase 1, glycogen synthase, phosphorylase, phosphorylase kinase, and laforin (37–39). The PTG family members display differential expression patterns in that PTG is expressed in all insulin-sensitive tissues, whereas GL is expressed mainly in the liver and GM is expressed in the muscle (40, 41). R6 displays a more ubiquitous expression pattern (42).

Each of the PTG family members was expressed as a Myc-tagged fusion protein in CHO-IR cells along with FLAG-tagged laforin. Laforin was immunoprecipitated from these cells and analyzed for the association of PTG family members using antibodies directed toward the Myc epitope. All of the PTG family members were detectable in CHO-IR cell extracts except GM, which was expressed at such low levels that the fusion protein could only be detected after immunoprecipitation (Fig. 2B, left panels). PTG, GL, and R6 all co-immunoprecipitated with laforin, with R6 being the most robust (Fig. 2B, right panel). GM could not be detected in the co-immunoprecipitate, possibly due to its low expression level (Fig. 2B, left panel). PTG was further evaluated as a substrate for laforin, as described above for GS, and similar negative results were obtained (data not shown).

We utilized similar strategies to test the ability of laforin to dephosphorylate other enzymes involved in glycogen metabolism, including malin (13, 14), glycogen branching enzyme (43), protein phosphatase 1 inhibitor 2 (44), β-catenin (45), and the AMPKα/β subunits (46) (data not shown). Our conclusion is that although laforin is found in a complex with many proteins involved in glycogen metabolism, it does not dephosphorylate any of the other proteins associated with glycogen metabolism that were tested. These results are in
agreement with multiple studies that have failed to find any changes in the activities of enzymes associated with glycogen metabolism in LD patients (47–49).

Laforin Dephosphorylates a Complex Polysaccharide—CBM20 domains are commonly found in a variety of glycosylhydrolases in plants, fungi, and bacteria. The vast majority of enzymes that contain a CBM20 domain, such as α-amylase or glucoamylase, use this domain to bind directly to the carbohydrate and then enzymatically act on the sugar itself (31). As previously mentioned, LBs are structurally amylopectin-like in nature and are phosphorylated. Therefore, we hypothesized that laforin might dephosphorylate the LB itself. Since we have been unable to obtain enough pure LB material to test as a substrate, we turned to its closest equivalent, plant starch (25, 28, 29). In particular, potato amylopectin is phosphorylated on approximately 1 in 300 glucose residues (27). Phosphorylation occurs on either the C-3 (30–40% of the time) or the C-6 (60–70% of the time) position of the glucose residue and is important in starch metabolism (50–52). Recently, a putative laforin functional homologue has been reported in plants called starch excess 4 (SEX4) (53, 54). SEX4 has a putative phosphatase domain followed by a starch-binding domain (53, 54). Plants also express a protein kinase known as R1 that is responsible for phosphorylating glucose residues in amylopectin (55, 56). To date, our data base searches have not yielded a eukaryotic R1 equivalent. Although the roles that R1 and SEX4 play in the storage and utilization of plant starch are currently not well understood, they are both clearly involved in starch metabolism (50, 57). In fact, SEX4 mutant plants display a starch excess phenotype reminiscent of the accumulation of LBs in Lafora disease (53).

Since LBs are most similar to amylopectin and both are reportedly phosphorylated, we tested potato amylopectin as a potential substrate for laforin using the malachite green assay. This assay is highly sensitive for detecting inorganic phosphate (30). WT laforin displayed robust phosphatase activity toward potato amylopectin (Fig. 3). This activity is not the result of a co-purifying enzyme, since catalytically inactive laforin (C/S) is not able to catalyze this reaction. In addition, laforin does not remove phosphate residues from glycogen in our assay. This is most likely a result of the fact that normal cellular glycogen does not contain an appreciable quantity of phosphate residues, and our assay conditions may not be able to detect this low a level of phosphate release. Because of the unusual nature of this activity, we also tested SEX4, the plant protein that contains a phosphatase and starch-binding domain (53), for its ability to dephosphorylate amylopectin.3 In light of these results, we hypothesize that the role of laforin is to maintain proper glycogen metabolism by removing phosphate residues during either glycogen synthesis or degradation. In the absence of laforin, we predict that LBs, unlike glycogen, would contain phosphate; indeed, this has been reported on several occasions in the literature (26, 58, 59).

Laforin Is Unique in Its Ability to Dephosphorylate Amylopectin—Due to the unusual nature of this activity, we sought to ascertain if other active phosphatases could indiscriminately dephosphorylate amylopectin. In order to test this hypothesis, we selected several different types of phosphatases for our analysis: PTPMT1, a dual specificity phosphatase that prefers phosphatidylinositol 5-phosphate as its substrate (60); TCPTP, a phosphotyrosine-specific phosphatase (61); protein phosphatase 1, a very active serine/threonine phosphatase (62); alkaline phosphatase (AlkP), a more nonspecific phosphatase that can dephosphorylate DNA as well as protein substrates (63); VHR, a dual specificity phosphatase (64); and dullard, a phospho-Ser/Pro-directed phosphatase (65). In each case, the purified recombinant phosphatases were capable of utilizing pNPP as a

3 M. S. Gentry and J. E. Dixon, unpublished results.
substrate (Fig. 4A). However, only laforin was capable of removing phosphate from amylopectin (Fig. 4B). As mentioned previously, since amylopectin can be phosphorylated on both the 3'- and 6'-OH groups, the substrate is heterogeneous. This precludes us from undertaking more detailed analyses to determine $K_m$ or $K_{cat}$ values for this substrate. To obtain an assessment of the relative activity of laforin toward amylopectin, we generated a relative measure of an enzyme’s ability to remove phosphate from amylopectin versus its activity against pNPP (Fig. 4B, numbers above bars). Using this criterion, laforin is 50–700 times more efficient at removing phosphate from amylopectin than the other phosphatases. This suggests that removal of phosphate from amylopectin is not a property common to phosphatases in general but rather requires a specific orientation of the phosphatase active site to the phosphorylated sugar. Roach and co-workers recently measured the activity of laforin against pNPP in the presence of glycogen and amylopectin (66). They noted that the addition of glycogen to the reaction caused potent inhibition of pNPP hydrolysis and that the less branched glucose polymers, amylopectin and amylose, were more potent inhibitors. They hypothesized that laforin undergoes a conformational change that blocks its active site upon binding a complex carbohydrate. In light of our results, this inhibition may more likely be a result of competition for the active site of laforin.

**VHR Containing a CBM Is Not Able to Dephosphorylate Amylopectin**—Our experiments utilizing amylopectin were performed *in vitro*, and the possibility existed that since laforin was the only phosphatase tested that contained a CBM, it was the only one capable of binding the potential substrate. It occurred to us that attaching the CBM of laforin to another phosphatase would allow the fusion protein to bind to amylopectin, possibly conferring activity onto its phosphatase domain. In order to test this hypothesis, we aligned the laforin phosphatase domain with VHR and fused the aligned portion in frame to the laforin CBM (Fig. 5A). We then expressed and purified the fusion protein (CBM-VHR) from bacteria and tested it for phosphatase activity against pNPP and amylopectin. CBM-VHR retains ~10% of the wild type VHR activity when pNPP is used as a substrate and is capable of binding glycogen (data not shown). However, the CBM-VHR fusion protein was not capable of dephosphorylating amylopectin (Fig. 5B). Thus, we conclude that the active site of laforin is unique in its ability to utilize a phosphorylated complex carbohydrate as a substrate.

Although we cannot preclude the possibility that laforin also has a proteinaceous substrate, we have demonstrated that laforin displays robust activity against the phosphorylated complex carbohydrate amylopectin. Moreover, we demonstrate that activity against amylopectin is not a common property of phosphatases in general. Although it was previously reported that cellular glycogen contains phosphate mono- and diester substitutions at the C-6 position of some glucose units, there is no compelling explanation for the function of phosphate on glycogen (67). However, it is possible that glycogen can serve as a substrate for a glucose-phosphate-transferring enzyme as suggested by Lomako *et al.* (68). Additionally, these researchers have postulated that the phosphate content could be linked to branching and glycogen synthesis. Indeed, there is precedence for this idea in plants, where a tight relationship between starch phosphorylation and the degree of starch branching exists (51).

Our hypothesis is that laforin removes the phosphate monoesters from glycogen, allowing glycogen metabolism to proceed normally. Therefore, in the absence of laforin, glycogen accumulates more phosphate residues and longer unit chains, eventually forming LBs that resemble insoluble amylopectin. Whether laforin functions during glycogen synthesis or breakdown, our results raise the provocative and unexpected finding that laforin is capable of removing phosphate monoester residues from complex carbohydrates. Although unexpected, our data point to a heretofore overlooked aspect of glycogen metabolism that may be critical in understanding the molecular etiology of Lafora disease.

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