The phosphatase laforin removes phosphate groups from glycogen during biosynthetic activity. Loss-of-function mutations in the gene encoding laforin is the predominant cause of Lafora disease, a fatal form of progressive myoclonic epilepsy. Here, we used hybrid structural methods to determine the molecular architecture of human laforin. We found that laforin adopts a dimeric quaternary structure, topologically similar to the prototypical dual specificity phosphatase VH1. The interface between the laforin carbohydrate-binding module and the dual specificity phosphatase domain generates an intimate substrate-binding crevice that allows for recognition and dephosphorylation of phosphomonoesters of glucose. We identify novel molecular determinants in the laforin active site that help decipher the mechanism of glucan phosphatase activity.

The origin of glycogen phosphorylation in humans is unknown. Although plants possess several known starch kinases (11), a dedicated glycogen “kinase” has not been identified in metazoans. It was suggested (12) that glycogen accumulates phosphate at positions C2 and C3 of glucose as a result of the normal biosynthetic activity of glycogen synthase, which erroneously incorporates one phosphate moiety every ~10,000 glucose units. This finding was challenged by a recent report (2) that identified C6 as another major phosphorylation site in human glycogen. Because the mechanism proposed for glycogen phosphorylation by glycogen synthase cannot explain phospho-esterification at position C6 (12), alternative sources of glycogen phosphorylation are likely to exist. Laforin is the only glycogen phosphatase encoded in the human genome (13), and it is conserved in all vertebrates but absent in most invertebrate organisms, including model organisms such as yeast, flies, and worms (14). In this study, we used hybrid structural methods to characterize the three-dimensional organization of human laforin and to shed light on the mechanisms and regulation of glucan phosphatase activity.

**Experimental Procedures**

*Biochemical Techniques—*Human laforin was cloned in vector pQE-80L lacking the N-terminal His tag. Laf-DSP (residues 150–331) and a synthetic gene encoding Laf-CBM (residues 1–137) (GenScript) were cloned in an engineered pET-28a vector containing an N-terminal maltose-binding protein gene. Point mutants C266S, C329S, C169S, R272A, R171A, D235A, and D197A were generated by site-directed mutagenesis. All proteins were expressed in Escherichia coli strain BL21 (DE3) RIL or Express I1 (New England Biolabs) cells by inducing at 20 °C for 12–16 h with 0.25 mM isopropyl 1-thio-β-D-galactopyranoside. Cell pellets were dissolved in Lysis buffer containing 50 mM HEPES, pH 7.5, 500 mM NaCl, 1 mM tris(2-carboxyethyl)phosphine (TCEP), 10% glycerol, 0.4% n-dodecyl β-D-maltoside, 1 mg ml⁻¹ lysozyme, 80 μg ml⁻¹ DNase, 1.0 mM phenylmethylsulfonyl fluoride and were disrupted by sonication. Recombinant proteins were purified on amylose beads (New England Biolabs), eluted from beads with 90 mM α-cyclodextrin, and further purified on a Superose 12 gel filtration column (GE Healthcare) in GF1-buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM TCEP, 2% glycerol), as described previously (15).

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**Background:** Laforin is an essential glycogen phosphatase often mutated in Lafora disease.

**Results:** Human laforin adopts a dimeric quaternary structure.

**Conclusion:** Dimerization generates a unique active site crevice essential to recognize and dephosphorylate glycogen.

**Significance:** We derived a complete structural model of human laforin using hybrid structural methods.
Laforin (Protein Data Bank 3RGO) as the initial search model, as implemented in phenix.mr_rosetta (32). This solution was subjected to autobuilding and refinement in Phenix (33) and COOT (34). The final model has an $R_{work} / R_{free}$ of 18.8/21.9% (using all data between 15 and 2.30 Å resolution) and excellent geometry (Table 1). The sulfhydryl groups of Cys-250, Cys-278, and Cys-329 are covalently bound β-mercaptoethanol (data not shown).

RESULTS

“Divide and Conquer” Approach to Study Laforin Structure—Laforin has a bipartite organization that consists of an N-terminal CBM spanning residues 1–130 and a C-terminal DSP domain (residues 150–331) (Fig. 1A). Although laforin is partially membrane-bound in vivo (35), the protein can be extracted from lipids using nonionic detergents and solubilized with carbohydrates. In vitro, purified laforin is stabilized upon binding to carbohydrates that enhance its solubility by burying hydrophobic residues in the CBM (36). We measured an apparent melting temperature ($T_{m,app}$) of 62 °C for purified laforin bound to α-cD and 68 °C in the presence of maltose; on the contrary, removing carbohydrates by prolonged dialysis reduced laforin stability and made the protein aggregation-prone (Fig. 1B). Because laforin failed to crystallize as a full-length protein, we expressed and purified individual DSP and CBM domains in large quantity for crystallization and studied the full-length laforin in solution using SAXS, AUC-SV, and phosphatase assay.

Crystal Structure of Laforin DSP Domain—We crystallized an inactive mutant (C266S) of laforin DSP (laf-DSP) spanning residues 150–331, and we determined its structure to an $R_{work} / R_{free}$ of 18.8/21.9%, at 2.3 Å resolution (Table 1). Laf-DSP consists of a central five-stranded β-sheet ($β_1$ to $β_5$) sandwiched between two clusters of seven α-helices ($α_{1}$ to $α_{3}$ and $α_{4}$ to $α_{7}$), similar to the prototypical DSP VH1 (Fig. 1C) (21). The laforin catalytic triad consists of Cys-266 (the catalytic residue), Arg-272 (the general base), and Asp-235 (the general acid); the first two residues are part of a phosphate-binding loop, or “P-loop” (residues 265–272), whereas Asp-235 is located in a separate loop, the “general acid” loop (residues 230–238). Secondary structure alignment of laf-DSP with the starch phosphatase Sex4 (37) (root mean square deviation of 2.4 Å) and the phosphoinositide phosphatase PTEN (38) (root mean square deviation of 2.6 Å) reveals a superimposable position of the catalytic cysteine and general base, whereas the laforin general acid Asp-235 is shifted away from the active site (7.9 Å versus ~3.5 Å) (Fig. 1C). The open conformation of the laforin general acid loop is stabilized by the bulky side chain of Tyr-304 that projects from the surface of the “TI-loop” (residues 296–307) (Fig. 1D). The open conformation of the laforin general acid loop is stabilized by the bulky side chain of Tyr-304 that projects from the surface of the “TI-loop” (residues 296–307) (Fig. 1D).

Crystallographic Methods—Laf-DSP was crystallized using 0.10 M lithium sulfate, 0.05 M sodium cacodylate, pH 6.5, 0.1 M NaCl, 10 mM TCEP, at 18 °C. Crystals were cryo-protected with 30% ethylene glycol, flash-frozen in liquid nitrogen, and diffracted at beamlines X6A and X29 at the National Synchrotron Light Source. Data reduction was done with the HKL2000 (Table 1) (31), and the structure was solved by molecular replacement and structure modeling in Rosetta using PTPMP1 (32). The theoretical solution scattering curves were calculated using CRYSOL (30). Theoretical solution scattering curves were calculated using Ab initio (31), and the structure was solved by molecular replacement and structure modeling in Rosetta using PTPMP1 (32). The theoretical solution scattering curves were calculated using CRYSOL (30). The correlation coefficient between the SAXS envelope and atomic model is 0.845 for laf-CBM and 0.806 for dimeric laforin.

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similar in topology to the catalytic triad. The two triads appear to cross-talk structurally in two respects. First, Cys-169 and the catalytic Cys-266 are equidistant (∼3 Å) from a histidine (His-265), which in classical phosphotyrosine phosphatases is known to decrease the active site cysteine pKa, promoting formation of a thiolate intermediate at physiological pH (39). Second, Asp-197 makes a bidentate salt bridge with the guanidinium group of the general base Arg-272. Thus, the crystallographic structure of laf-DSP reveals a classical phosphotyrosine phosphatase-fold characterized by an unusually broad and shallow active site crevice and an open conformation of the general acid loop.

SAXS-restrained Model of Laforin CBM Bound to α-cD—Unlike the phosphatase domain, we were not able to crystallize laf-CBM. We then generated a homology model of this conserved domain (Fig. 2A) using the prediction server I-TASSER (40), which uses multiple-threading alignments and iterative template fragment assembly simulations. We also measured the SAXS profile of human laf-CBM in the presence of 10 mM α-cD and calculated an ab initio SAXS envelope using scattering data at 3.2 mg ml⁻¹ concentration. Laf-CBM SAXS envelope is shaped like a “pear,” with a tapered and a bulgy end, and it is slightly curved when rotated by 90° (Fig. 2A). Laf-CBM homology model was docked inside the SAXS density, and the
position of α-cD was determined by superimposing laf-CBM to the structure of the *Aspergillus niger* CBM20 bound to β-cD (Protein Data Bank code 1ACZ) (41). The composite laf-CBM model bound to α-cD was then refined against the SAXS envelope using low resolution real space refinement. The excellent agreement of distance distribution functions (P(r)) calculated from experimental SAXS data and laf-CBMα-cD complex (Fig. 2B) suggest our model of laf-CBM is reasonably accurate.

**Solution Structure of Human Laforin**—To investigate the oligomeric state of human laforin, we subjected purified laforin to AUC-SV analysis in a range of concentrations (0.25–0.7 mg ml⁻¹). At physiological salt and in the presence of reducing agents, laforin migrated as a homogenous species with an apparent sedimentation coefficient (s*) of 3.85 S, corresponding to a dimer of ~71.1 kDa (expected mass of 74.2 kDa) (Fig. 3A and Table 2). A C329S mutant of laforin had nearly identical s* (Table 2), ruling out the involvement of Cys-329 in dimerization (42). Likewise, SAXS spectra measured at a concentration of laforin between 0.7 and 5.0 mg ml⁻¹ gave a gyration radius and maximum diameter of 33.4 and 115.0 Å, respectively, remarkably close to the Stokes radius calculated from SV analysis and fully consistent with a dimeric quaternary structure (Table 2).

To visualize the structural organization of dimeric laforin, we generated an *ab initio* shape reconstruction from scattering data at 2.0 mg ml⁻¹. Laforin’s SAXS envelope revealed an ellipsoidal shape characterized by three lobes of density (Fig. 3B), which we interpreted using the structures of laf-DSP and laf-CBM described above. The external lobes in the SAXS envelope are rotated by 75° with respect to each other and each fit one DSP domain; the central lobe instead is too large for one DSP but accommodates a dimer of laf-CBMs arranged in a parallel fashion (Fig. 3B). This quaternary structure yields excellent agreement of P(r) functions calculated from experimental SAXS data and the proposed model (Fig. 3C). Some ambiguity remains in the way laf-DSPs connect to CBMs that could be swapped, like in VH1 (18, 21), or juxtaposed, although either topology generates an equivalent quaternary structure. Mapping sugar-binding residues in this model using the structure of Sex4 bound to maltoheptaose (37) as a template and knowledge of LD mutations that disrupt sugar binding (8) identified two contiguous clusters of amino acids, one in the CBM (Trp-32, Phe-84, Trp-85, Lys-87, Trp-99, and His-105) and the other in the DSP (Gln-200, Asn-201, Met-236, Arg-241, Lys-299, and Arg-300). The two clusters face each other in our model (Fig. 3D) generating a narrow pocket that sandwiches a bound glucan, presenting it to the phosphatase active site. This model also explains the high affinity of laforin for glucan chains and its preference for phosphoglucose over peptidic substrates.

**Dimerization Triggers Laforin Catalytic Activity**—To dissect the structural basis for glucan phosphatase activity, we mutated each of the residues in the laforin catalytic triad (Cys-266, Arg-272, and Asp-235) and second triad (Cys-169, Arg-171, and Asp-197) (Fig. 1E) and we measured the phosphatase activity of these mutants toward potato amyllopectin and OMFP. Although laf-DSP was completely inactive, dimeric wild type (WT) laforin efficiently dephosphorylated amylopectin (Fig. 4A) and OMFP (k_{cat}/K_m = 1.90 × 10^{8} M^{-1} s^{-1} and K_m = 520 ± 2520 s⁻¹) (Table 2), suggesting that the presence of the DSP domain is a prerequisite for phosphatase activity.

**TABLE 1**

| Crystallographic data collection and refinement statistics |
|-----------------------------------------------------------|
| Data collection                                           |
| Beamline                                                  |
| X29                                                       |
| Wavelength (Å)                                           |
| 1.07                                                      |
| Space group                                               |
| I 4                                                       |
| Cell dimensions                                          |
| a, b, γ (Å)                                               |
| 123.9, 123.9, 160.7                                       |
| α, β, γ (%)                                               |
| 90.0, 90.0, 90.0                                          |
| Unique reflections                                       |
| 52,012                                                    |
| Completeness, %                                          |
| 97.1 (88.3)                                               |
| Resolution (Å)                                           |
| 15.0-2.3 (2.4-2.3)                                        |
| R_{exp}                                                  |
| 9.6 (53.0)                                                |
| Redundancy                                               |
| 2.9 (2.2)                                                 |
| Wilson B Factor                                          |
| 19.3 (1.9)                                                |
| No. of reflections                                       |
| 51,890                                                    |
| R_{work}/R_{free}                                         |
| 18.8/21.9                                                 |
| No. of protein residues                                   |
| 716                                                       |
| No. of solvent molecules                                 |
| 243                                                      |
| No. of β-ETOH/ions                                       |
| 12/4                                                      |
| B-Factor                                                 |
| Protein atoms                                            |
| 59.7                                                     |
| Sulfate ions                                              |
| 55.9                                                     |
| Waters                                                    |
| 54.1                                                     |
| Ramachandran plot (%) core/allowed/generally allowed/disallowed |
| 89.5/9.9/0.6/0.0                                          |
| Root mean square deviations from ideal                   |
| Bond lengths (Å)                                         |
| 0.008                                                    |
| Bond angles (%)                                          |
| 1.159                                                    |

*The value was calculated using 1,969 randomly selected reflections.*

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**FIGURE 2.** SAXS-restrained model of laforin CBM bound to α-cD. A, *ab initio* SAXS reconstructions of laf-CBM (in gray) calculated from experimental scattering values at 3.2 mg ml⁻¹. Overlaid to the SAXS envelope is a homology model of human laf-CBM. B, agreement of P(r) functions calculated from experimental SAXS data (blue) and data calculated from the laf-CBM model (red). Inset shows experimental scattering data (blue) overlaid to the scattering curve calculated from the laf-CBM model (red).
**FIGURE 3. Quaternary structure of human laforin.**

A, sedimentation velocity profiles of human laforin at 20 °C. Top panel, raw absorbance at 280 nm plotted as a function of the radial position. Data at intervals of 60 min are shown as open circles for sedimentation at 35,000 rpm. Bottom panel, fitted distribution of s*.

B, *ab initio* SAXS reconstruction of dimeric laforin (light gray) displayed in side and top views overlaid to a pseudo-atomic model of the enzyme obtained by docking ribbon models of laf-DSP and laf-CBM inside the SAXS envelope.

C, agreement of P(r) functions calculated from experimental SAXS data (blue) and data calculated from the pseudo-model (red) of dimeric laforin. Inset, experimental scattering data (blue) overlaid to the scattering curve calculated from the laforin model (red).

D, a model of dimeric laforin (gray mesh) bound to α-cD (green); carbohydrate-binding residues projecting from laf-CBM (in yellow) and laf-DSP (cyan) (shown only for one active site), as well as Cys-266/Cys-169, and sulfate ions are shown as thick sticks. Underlined are residues mutated in LD (8).
phosphoenzyme intermediate, which decreases the Michaelis-Menten constant (defined as $K_m = (k_{-1} + k_2)/k_1$).

Next, we examined the effect of mutations in the second triad. Mutation of Cys-169 and Arg-171 reproducibly reduced glucan phosphatase activity by 6 and 12%, respectively (Fig. 4A), without decreasing laforin specificity ($K_m = 580 \pm 10 \mu M$ and $K_m = 400 \pm 20 \mu M$) and catalytic efficiency ($k_{cat}/K_m = 1.53 \times 10^8 M^{-1} s^{-1}$ and $k_{cat}/K_m = 2.70 \times 10^8 M^{-1} s^{-1}$) toward OMFP (Fig. 4B). In contrast, D197A completely disrupted laforin activity (Fig. 4, A and B) possibly because mutations of acidic residues stabilizing the general base severely affect catalytic activity (43). Importantly, none of these mutations reduced laforin structural stability, as assessed from their heat-induced denaturation curves (data not shown). Thus, mutations in the second triad also affect laforin phosphatase activity, suggesting an active involvement of these residues in glycogen dephosphorylation.

**DISCUSSION**

Over a century after the discovery of LD (1), and nearly 2 decades since the identification of the laforin gene (4), we have derived a complete structural model of human laforin using hybrid structural methods. This structure provides clues to understanding laforin glycogen phosphatase activity, important to decipher the molecular etiology of LD.

The oligomeric state of human laforin has been controversial with reports of laforin forming a monomer (44), a dimer (45, 46), and even a cross-linked dimer (42). Combining the power of AUC-SV with SAXS and folding studies, we found that laforin folds into a stable homodimer in a range of concentrations between 0.25 and 5.0 mg ml$^{-1}$ (Fig. 3, A and B). This supports previous findings by Liu et al. (45) and contradicts a recent report that laforin is monomeric based solely on size exclusion chromatography (44). Our structural analysis reveals that the laforin quaternary structure is stabilized by dimerization of its N-terminal CBM (Fig. 3B). This is analogous to how VH1 assembles into a dimer by swapping an N-terminal helix (18, 21). As observed for VH1 (18, 21), and reported for laforin (45),
dimerization is essential for catalytic affinity. Whereas the isolated DSP domain of laforin is inactive in vitro, the dimeric full-length protein is highly active toward glucans and OMFP (Fig. 4, A and B). We propose that dimerization leads to laforin activation in two ways. First, it induces intramolecular contacts between DSP and CBM that force the acidic loop closer to the active site, in a conformation poised for acid-base catalysis. Second, it closes the sugar-binding pocket positioning a bound glucan between two clusters of carbohydrate-binding residues, thereby reducing the rate of substrate dissociation from the active site (Fig. 3D). However, swapping laf-DSP with VH1-related (VHR) results in a phosphatase inactive toward polyglucosan (47), suggesting that CBM-induced dimerization and activation of phosphatase activity are necessary but not sufficient to promote laforin glucan phosphatase activity, which depends on unique structural determinants in laf-DSP. In a quest for these determinants, we identified a second surface-exposed pocket next to the laforin active site lined by three residues strikingly similar to the catalytic triad (Fig. 1E). Our mutational analysis suggests the second triad is also important for laforin glucan phosphatase activity; disrupting either Cys-169 or Arg-171 results in a modest yet reproducible decrease in glucan phosphatase activity, although mutation of Asp-197 completely disrupts phosphatase activity (Fig. 4A). Although it may be argued that most point mutations of surface-exposed residues next to the active site destabilize substrate binding, mutations at Cys-169 and Arg-171 did not decrease laforin affinity for OMFP (Fig. 4B). We propose that laforin second triad functions in synergy with the catalytic triad possibly by dephosphorylating different phosphomonoesters of glucose. Unfortunately, the amylopectin phosphatase assay is poorly sensitive, and unlike glycan, amylopectin lacks phosphate groups at position Cys-2 (2, 48), arguing that laforin-C266S, thus far thought to be a catalytically dead mutant, could indeed harbor residual activity. To corroborate the dichotomy between laforin activity toward amylopectin in vitro and glycogen phosphatase activity in vivo, a missense point mutation at position Arg-171 causes LD in vivo (8), whereas an alanine mutation at this position decreased amylopectin dephosphorylation by only 12% (Fig. 4A). Thus, even a modest deficiency in phosphatase activity in vitro may result in devastating changes in brain glycogen phosphorylation in vivo. Our structural finding of a second triad immediately next to the laforin active site may help resolve a major conundrum in the field raised by a recent well-founded report that overexpression of laforin-C266S in laforin-deficient mice wholly rescues LD (49), suggesting that the phosphatase activity of laforin, its only known enzymatic activity, is irrelevant. We propose that laforin’s second triad harbors residual catalytic activity, which sufficiently dephosphorylates glycogen when laforin-C266S is overexpressed (49). The absence of laforin-C266S in vitro phosphatase activity could imply that the second triad requires conditions that are only maintained in vivo, in nondividing cells like neurons.

In conclusion, the long-sought structure of human laforin presented in this study sheds light on the molecular basis for glycogen dephosphorylation and sets the ground for future investigations to validate a putative second phosphatase active site.

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