A Unique Carbohydrate Binding Domain Targets the Lafora Disease Phosphatase to Glycogen*

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Lafora disease (progressive myoclonus epilepsy of Lafora type) is an autosomal recessive neurodegenerative disorder resulting from defects in the EPM2A gene. EPM2A encodes a 331-amino acid protein containing a carboxyl-terminal phosphatase catalytic domain. We demonstrate that the EPM2A gene product also contains an amino-terminal carbohydrate binding domain (CBD) and that the CBD is critical for association with glycogen both in vitro and in vivo. The CBD domain localizes the phosphatase to specific subcellular compartments that correspond to the expression pattern of glycogen processing enzyme, glycogen synthase. Mutations in the CBD result in mis-localization of the phosphatase and thereby suggest that the CBD targets laforin to intracellular glycogen particles where it is likely to function. Thus naturally occurring mutations within the CBD of laforin likely result in progressive myoclonus epilepsy due to mis-localization of phosphatase expression.

Lafora disease (OMIM 254780) is an autosomal recessive neurodegenerative disorder. It accounts for a subset of severe epilepsies with myoclonic, tonic seizures and progressive neurologic deterioration. The disease usually occurs between the ages of 7 and 20 and results in death within 10 years. Lafora disease is characterized by the accumulation of intraneuronal periodic acid-Schiff-positive cytoplasmic inclusion bodies (Lafora bodies), which contain 80–93% polyglucosan (1, 2). Lafora bodies also develop in brain, liver, skin, kidney, skeletal, and cardiac muscle, and biopsy of axillary skin provides a reliable diagnosis for Lafora disease (3–6).

Minassian et al. (7) and Serratos et al. (8) independently identified the gene mutated in Lafora disease to be present on chromosome 6q24. This chromosome localization distinguishes EPM2A from other genes related to Lafora disease. The EPM2A gene is located on chromosome 6q24. This chromosome localization distinguishes EPM2A from other genes related to Lafora disease. The EPM2A gene encodes a 331-amino acid protein containing a carboxyl-terminal phosphatase catalytic domain. We demonstrate that the EPM2A gene product also contains an amino-terminal carbohydrate binding domain (CBD) and that the CBD is critical for association with glycogen both in vitro and in vivo. The CBD domain localizes the phosphatase to specific subcellular compartments that correspond to the expression pattern of glycogen processing enzyme, glycogen synthase. Mutations in the CBD result in mis-localization of the phosphatase and thereby suggest that the CBD targets laforin to intracellular glycogen particles where it is likely to function. Thus naturally occurring mutations within the CBD of laforin likely result in progressive myoclonus epilepsy due to mis-localization of phosphatase expression.

Lafora disease from the progressive myoclonus epilepsy of Unverricht-Lundborg type (21q22.3). Positional cloning of the EPM2A (epilepsy of progressive myoclonus type 2) gene revealed an encoded protein product of 331 amino acids containing a dual specificity protein phosphatase catalytic active site motif, HCXGXXRS/T (9, 10). A total of 30 different disease-related mutations have been described in the EPM2A gene (11, 12), of which 12 cause missense mutations (Fig. 1A). In this study, we show that the NHE terminus of laforin contains a carbohydrate binding domain that targets the phosphatase to glycogen when it is likely to function. Mutations within the CBD abolish the binding of laforin to glycogen, and this is likely to be the cause of some forms of progressive myoclonus epilepsy.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Laforin was amplified from a human muscle cDNA library (CLONTECH) by PCR and cloned into BamHI and HindIII sites of the bacteria expression plasmid pET21a (Novagen) to produce a recombinant protein with a COOH-terminal 6-histidine tag. The cDNA of laforin was also cloned into a mammalian expression vector pcDNA3.1NF (13) by PCR. Fusion proteins expressed from vector pcDNA3.1NF contain an NH2-terminal M2-FLAG epitope of 8 amino acid residues. To produce a fusion protein with a COOH-terminal enhanced green fluorescence protein (EGFP), the cDNA sequence of laforin was excised from pET21a-Laf with NheI and HindIII and ligated to the same sites of pEGFP-N1 (CLONTECH). All site-directed mutations were confirmed by nucleotide sequencing. The cDNA of human glycogen synthase was amplified from the human muscle cDNA library (CLONTECH) and contained a COOH-terminal Myc epitope of 10 amino acid residues.

Expression and Purification of Recombinant Protein—Recombinant proteins were expressed in Escherichia coli BL21 (DE3) Codonplus cells (Stratagene). The expressed proteins were purified using Ni2+–agarose (Qiagen) as described previously (14).

Cell Culture and Transfection—HEK 293 cells and COS1 cells were grown in modified Eagle's medium supplemented with fetal bovine serum (10% v/v), penicillin (100 units/ml), streptomycin (100 mg/ml), and l-glutamine (2 mM) (Invitrogen). Transfections with the cDNA constructs were carried out using FuGENE 6 (Roche Diagnostics).

Coexpression with Glycogen—For in vitro experiments, 0.5 μg of purified recombinant protein was incubated in 1 ml of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) 2-mercaptoethanol, 0.1 mg/ml bovine serum albumin containing 10 μg/ml glycogen (Roche Diagnostics) at 4 °C for 30 min. After centrifugation at 100,000 × g for 90 min, the supernatant and the pellet fractions were collected and subjected to Western blot analysis using anti-polyhistidine antibody His probe (H-15) (Santa Cruz Biotechnology). For in vivo experiments, HEK 293 cells were transiently transfected and after 24 h, the cells were washed with cold PBS three times and harvested in hypotonic buffer consisting of 20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 200 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 μg/ml aprotinin, leupeptin, and pepstatin. The cells were then lysed on ice using Dounce homogenizer, and the cell lysates were centrifuged by centrifugation at 10,000 × g for 10 min. After incubating at 30 °C for 30 min in the presence or absence of 5 units/ml of α-amylase (Sigma), the cell lysates were ultracentrifuged at 100,000 × g for 90 min, and the supernatant and pellet fractions were collected and subjected to Western blot analysis using anti-FLAG M2 antibody (Sigma).

Immunoprecipitation—24 h after transfection, HEK 293 cells were washed with ice-cold PBS three times and harvested in ice-cold lysis buffer consisting of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, leupeptin, and pepstatin. The cells were lysed at 4 °C
by constant agitation for 30 min, and then the cell lysates were cleared by centrifugation at 10,000 \( \times g \) for 10 min. The supernatants were subjected to binding with prewashed anti-FLAG M2 affinity resin (Sigma) at 4 \(^\circ\)C for 4 h by constant rotation (using 25 \( \mu \)l of resin slurry/10\(^6\) cells). Then the resins were pelleted by centrifugation at 500 \( \times g \) for 1 min and washed three times with 1 ml of lysis buffer without 1% Triton X-100. The resulting resins were subjected to phosphatase activity assay and Western blot analysis.

**Western Blot**—The samples were analyzed on 12% SDS-polyacrylamide gels and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore). The membranes were probed with appropriate first antibodies and horseradish peroxidase-conjugated secondary antibodies.

**Phosphatase Activity Assay**—Para-nitrophenylphosphate (pNPP) assays were carried out at 30 \(^\circ\)C as described previously (15, 16), using 0.1 \( \mu \)g of recombinant protein.

**Subcellular Localization of Laforin Proteins**—COS1 cells were transfected with cDNA constructs of laforin-EGFP fusion proteins. After 24 h, the cells were washed with ice-cold PBS three times and fixed in 4% paraformaldehyde at room temperature for 10 min. For COS1 cells co-transfected with pEGFP-Lafl and pcDNA4/Myc-GS, after fixation with 4% paraformaldehyde, the cells were permeabilized in methanol at –20 \(^\circ\)C for 5 min. Then the cells were washed three times with PBS and blocked in 3% bovine serum albumin for 30 min at room temperature, followed by incubating with mouse anti-Myc antibody (Santa Cruz Biotechnology) and Texas red anti-mouse IgG (Vector Laboratories) at room temperature for 1 h, respectively. The Myc-tagged glycogen synthase kinase 3β was visualized as red fluorescence under fluorescence microscopy, while the laforin EGFP fusion proteins were visualized as green fluorescence.

**RESULTS AND DISCUSSION**

**Laforin has an NH\(_2\)-terminal CBD**—We cloned the laforin cDNA from a human muscle library (CLONTECH). The sequence of the coding region was identical to that published by Ganesh et al. (10). Analysis of the protein conserved domain data base (CDD; www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) revealed that laforin contained a putative starch binding domain (CBD-4), encompassing the NH\(_2\)-terminal 116 amino acids (Fig. 1B). CBD-4 is found in a variety of glycosylhydrolases from bacteria and fungi (17–19), where the function of CBD-4 is to bind polysaccharide substrates prior to cleavage.

To investigate the function of the CBD of laforin, several point mutations were created at the CBD-4 invariant residues (Fig. 1B) and their effects on both phosphatase activity and carbohydrate binding examined. Trp32 was mutated to Gly (W32G), since this is a mutation that is also found in Lafora disease (11, 12). A, sequence alignment of laforin with structurally defined CBD-4 domains from glycosylhydrolases. Conserved amino acids for CBD-4 domains identified in the conserved domain data base (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) are in red and the invariant residues highlighted. Lafora disease-related missense mutations (above) are shown for the laforin CBD-4 domain. Proteins and their corresponding species are listed below, along with their protein data bank file names or Swiss-Prot/TrEMBL accession numbers: Laforin H.s., Homo sapiens, TrEMBL: O95278; CDGT B.c., cyclodextrin glucosyltransferase from Bacillus circulans, protein data bank: 1DIJ; AMY B.s., α-amylase from Bacillus stearothermophilus, protein data bank: 1QHOA; AMY P.s., α-amylase from Pseudomonas stutzeri, protein data bank: 1GCY; CDGT B.c., cyclodextrin glucanotransferase from B. stearothermophilus, protein data bank: 1CYG; AMYG A.n., glucoamylase from Aspergillus niger, protein data bank: 1AC0; AMY T.c., α-amylase precursor from Thermomonomonas curvata, Swiss-Prot: P29575; CDG1 P.m., cyclomaltodextrin glucanotransferase precursor from Paenibacillus macerans, Swiss-Prot: P04830; AMYG A.a., glucoamylase precursor from Aspergillus oryzae, Swiss-Prot: P36914; AMYG N.c., glucoamylase precursor from Neurospora crassa, Swiss-Prot: P14804.

W32G and W32G/K87A, could not be detected in the glycogen-microsomal complexes, suggesting that they are not associated with the intracellular glycogen complexes. Only a very small fraction of K87A protein was present in the glycogen-microsomal complexes, and after α-amylase treatment all of the K87A protein was released into the cytosol. These results indicate that both the W32G and K87A mutations abolish the association of laforin with glycogen complexes.

Many proteins associate with intracellular glycogen complexes via interactions with glycogen binding proteins. For instance, the catalytic subunit of protein phosphatase 1 is associated with glycogen only when it binds to the glycogen-targeting regulatory subunits (21). To determine whether laforin interacts with an intermediate glycogen-binding protein or directly binds to glycogen, we carried out an in vitro glycogen binding experiment using protein-free glycogen and recombinant wild type and mutant laforin proteins. A His-tagged recombinant laforin was expressed in bacteria and purified on Ni\(^{2+}\)-agarose column. To determine whether the recombinant laforin was properly folded, the phosphatase activity was analyzed. The specific activity of the recombinant laforin toward pNPP was 1.34 \( \times 10^{-5} \) mol/mg/min at the optimum pH of 5.0.
anti-His6 antibody.

legend to

and the samples were ultracentrifuged and analyzed as described in the

"Experimental Procedures." Western blot (bottom) shows the immunoprecipitated proteins used in pNPP assays. B, amylase treatment removes laforin from glycogen-microsomal complexes. HEK 293 cells were transiently transfected as above, and the cell lysates were subjected to amylase treatment as described under “Experimental Procedures.” Petel (P) and the supernatant (S) are visualized by Western blot using anti-FLAG antibody. C, laforin directly binds to glycogen in vitro. Recombinant histidine-tagged laforin proteins were incubated with 10 mg/ml protein-free glycogen as described under “Experimental Procedures.” After ultracentrifugation at 100,000 × g for 90 min, proteins in the glycogen pellet (P) or supernatant (S) are visualized by Western blot using anti-His6 antibody. D, recombinant wild type laforin protein was incubated in the presence (+) or absence (−) of 10 mg/ml protein-free glycogen, and the samples were ultracentrifuged and analyzed as described in the legend to C.

Fig. 2. Laforin associates with glycogen in vivo and in vitro. A, phosphatase activities of wild type and mutant laforin proteins expressed in HEK 293 cells. HEK 293 cells were transiently transfected either with vector alone (pcDNA3.1NF) or with FLAG-tagged laforin constructs of wild type, protein tyrosine phosphatase active site mutant C266S, and CBD mutants W32G, K87A, or a double mutant W32G,K87A. Proteins were immunoprecipitated by anti-FLAG beads, and their activities toward pNPP (top) were assayed as described under “Experimental Procedures.” Western blot (bottom) shows the immunoprecipitated proteins used in pNPP assays. B, amylase treatment removes laforin from glycogen-microsomal complexes. HEK 293 cells were transiently transfected as above, and the cell lysates were subjected to amylase treatment as described under “Experimental Procedures.” Petel (P) and the supernatant (S) are visualized by Western blot using anti-FLAG antibody. C, laforin directly binds to glycogen in vitro. Recombinant histidine-tagged laforin proteins were incubated with 10 mg/ml protein-free glycogen as described under “Experimental Procedures.” After ultracentrifugation at 100,000 × g for 90 min, proteins in the glycogen pellet (P) or supernatant (S) are visualized by Western blot using anti-His6 antibody. D, recombinant wild type laforin protein was incubated in the presence (+) or absence (−) of 10 mg/ml protein-free glycogen, and the samples were ultracentrifuged and analyzed as described in the legend to C.

Under the same conditions, the CBD mutant protein W32G/K87A showed 75% activity of the wild type enzyme, while the C266S mutant was inactive. We were unable to purify the W32G mutant protein, because it was present in insoluble bacteria pellet. We incubated the purified recombinant proteins with glycogen at 4 °C for 30 min and then precipitated the glycogen particles by ultracentrifugation. Both wild type enzyme and the C266S mutant protein co-sedimented with glycogen, while the CBD mutant protein W32G/K87A remained in the supernatant (Fig. 2C). Wild type laforin protein did not exhibit any tendency to aggregate, excluding this a reason for its presence in the glycogen pellet (Fig. 2D). Thus our results suggest that laforin associates directly with glycogen, and mutations that disrupt the CBD of laforin abrogate the glycogen binding.

Based on our glycogen binding studies of laforin CBD domain, we developed a functional model by threading its amino acid sequence onto the crystal structure of cyclodextrin glycosyltransferase (protein data bank number: 2DJ). The modeled trisaccharide of glucose with α-1,4 linkages is depicted as a ball-and-stick diagram with carbons atoms in yellow and oxygens in red. Atoms of the invariant residues of the CBD of laforin are shown as balls-and-sticks with different colors corresponding to Fig. 1B: tryptophan residues are orange, Lys is blue, Pro is yellow, Phe is green, Leu is cyan, and Gly and Ala are gray. The invariant residue Gly is not shown, because it falls in one of the two sequence inserts of laforin that are not present in the sequence of cyclodextrin glycosyltransferase. The residues mutated in Laforin disease are shown as brown balls. Phe and Phe exist in the interior of the molecule, while Glu and Arg are surface-accessible. Trp is found in the carbohydrate binding pocket.

Fig. 3. Structural model of the CBD of laforin based on the crystal structure of cyclodextrin glycosyltransferase (protein data bank: 2DJ). The backbone of the modeled laforin structure is shown as a purple ribbon diagram created in Ribbons (28). The modeled trisaccharide of glucose with α-1,4 linkages is depicted as a ball-and-stick diagram with carbons atoms in yellow and oxygens in red. Atoms of the invariant residues of the CBD of laforin are shown as balls-and-sticks with different colors corresponding to Fig. 1B: tryptophan residues are orange, Lys is blue, Pro is yellow, Phe is green, Leu is cyan, and Gly and Ala are gray. The invariant residue Gly is not shown, because it falls in one of the two sequence inserts of laforin that are not present in the sequence of cyclodextrin glycosyltransferase. The residues mutated in Laforin disease are shown as brown balls. Phe and Phe exist in the interior of the molecule, while Glu and Arg are surface-accessible. Trp is found in the carbohydrate binding pocket.

Fig. 4. CBD domain targets laforin to the sites of glycogen complexes in cells. A, COS1 cells were transiently co-transfected with pEGFP-Laf and pcDNA4/Myc-GS. The expressed laforin-EGFP fusion protein and Myc-tagged glycogen synthase were visualized as described under “Experimental Procedures.” B, mutations in the CBD domain abrogate the laforin subcellular localization. COS1 cells were transiently transfected with laforin constructs fused to EGFP. Cells were fixed, and the green fluorescence fusion proteins were visualized under fluorescence microscope.

Based on our glycogen binding studies of laforin CBD domain, we developed a functional model by threading its amino acid sequence onto the crystal structure of cyclodextrin glycosyltransferase (protein data bank number: 2DJ) using program O (22). Our model predicts that the two invariant Trp residues in the CBD of laforin (Fig. 3, W32 and W99) directly interact with the polysaccharide in a manner similar to the two Trp residues (Trp and Trp) in cyclodextrin glycosyltransferase (Fig. 1B, CDGT). The invariant residue Lys is also predicted to directly interact with the carbohydrate via several hydrogen bonds. Lafora disease-related mutation of Trp to...
glycine (W32G) would disrupt the polysaccharide binding pocket and also potentially unfold the region immediately adjacent to the binding pocket. Our K87A mutation would also affect the carbohydrate binding by disrupting hydrogen bonds between the protein and carbohydrate. Other mutations found in patients with myoclonus epilepsy are also predicted to alter the ability of the protein to bind to glycogen. For example, F84L would affect the positioning of Trp85 in the polysaccharide binding pocket, while F88L would disturb the structural integrity of the polysaccharide binding site by altering the positioning of Lys87.

Laforin Is Localized to Cytoplasmic Glycogen Particles by Its CBD Domain—To study the subcellular localization of laforin, the full-length protein was fused to the NH2 terminus of EGFP. The laforin protein was localized to punctate cytoplasmic structures in transfected COS1 cells (Fig. 4, A and B). Glycogen synthase is known to localize to intracellular glycogen particles (23–26) and was used as a control for laforin expression. Expression of cDNA constructs pEGFP-Laf and pcDNA4/Myc-LS showed that laforin and glycogen synthase co-localize in the same punctate structures within the cytoplasm of transfected COS1 cells (Fig. 4A; laforin was shown in green fluorescence; glycogen synthase was shown in red fluorescence.). The subcellular distribution of the phosphatase-inactive laforin mutant proteins (C266S and D234A) showed the same punctate pattern as the wild type enzyme (Fig. 4B). In contrast, the CBD mutant laforins (W32G, K87A, W32G/K87A) distributed evenly throughout the cytoplasm and did not exhibit a punctate expression pattern (Fig. 4B), suggesting that the CBD domain is responsible for the specific subcellular localization of laforin. Taking together, our subcellular localization studies support the concept that laforin is targeted to intracellular glycogen particles by its CBD domain and mutations that disrupt carbohydrate binding abolish laforin targeting.

What is the in vivo substrate of laforin? Although there currently is no answer to this question, the clinical and genetic features of Lafora disease distinguish it from other well known glycogen storage diseases (27). Our work clearly demonstrated that laforin is targeted directly to glycogen. It would not be surprising that the substrate would also be directly involved in glycogen metabolism. The substrate likely contributes to the relative complexity of glycogen metabolism in vertebrates, since we have been unable to find laforin orthologues in worms relative complexity of glycogen metabolism in vertebrates, making it surprising that the substrate would also be directly involved in glycogen metabolism. For example, F84L in patients with myoclonus epilepsy are also predicted to alter the carbohydrate binding by disrupting hydrogen bonds adjacent to the binding pocket. Our K87A mutation would also affect the ability of the protein to bind to glycogen. For example, F84L would affect the positioning of Trp85 in the polysaccharide binding pocket, while F88L would disturb the structural integrity of the polysaccharide binding site by altering the positioning of Lys87.

In summary, our studies indicate that the laforin phosphatase contains an NH2-terminal CBD that targets the protein to glycogen and that mutations in CBD, including the W32G mutation found in Lafora disease, lead to mis-targeting of laforin. The characteristic histology of Lafora disease is an intraneuronal accumulation of Lafora bodies. The targeting of laforin to intracellular glycogen complexes suggests that it may act on proteins important in glycogen metabolism, which are also co-localized to similar sites within the cell. Mutations that disrupt the CBD of laforin would attenuate its localization to glycogen particles, where the substrate of laforin may reside. This provides an explanation for why some patients with mutations only in the CBD, but not in the phosphatase domain, develop Lafora disease.

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