Dimerization of Laforin Is Required for Its Optimal Phosphatase Activity, Regulation of GSK3β Phosphorylation, and Wnt Signaling*

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Epilepsy of progressive myoclonus type 2 gene A (EPM2A) encodes a dual specificity protein phosphatase called Laforin. Laforin is also a tumor suppressor that dephosphorylates GSK3β at the critical Ser9 position and regulates Wnt signaling. The epilepsy-causing mutations have a deleterious effect on phosphatase activity, regardless of whether they locate in the carbohydrate-binding domain (CBD) at the N terminus or the dual specificity phosphatase domain (DSPD) at the C terminus. How mutations outside the DSPD reduce the phosphatase activity of Laforin remains unexplained. Here we report that Laforin expressed in mammalian cells forms dimers that are highly resistant to SDS treatment. Deleting CBD completely abolished the dimerization and phosphatase activity of Laforin. Moreover, all of the naturally occurring Laforin mutations tested impaired laforin dimerization, GSK3β dephosphorylation at Ser9 and β-catenin accumulation in nucleus. Our results demonstrate a critical role of dimerization in Laforin function and suggest an important new dimension in protein phosphatase function and in molecular pathogenesis of Lafora’s disease.

Progressive myoclonus epilepsy or Lafora Disease (LD)4 is an autosomal recessive, fatal neurological disorder diagnosed with cytoplasmic inclusion bodies (Lafora Bodies) in the neuronal perikarya and dendrites, heart, muscle, skin, kidney, and liver (1) and is manifested with myoclonic and tonic-clonic seizures, progressively neurological degeneration, dementia and death within 10 years of onset. Loss-of-function mutations in two genes, EPM2A and EPM2B are identified as genetic causes of the diseases. EPM2A encodes a dual specificity protein phosphatase (DSP) known as Laforin (2, 3). EPM2B encodes an E3 ubiquitin ligase named Malin that ubiquitinates and then degrades the Laforin (4, 5). The Lafora bodies consist of 80–93% polyglucosan that is confirmed to be a long-stranded, less-branched and starch-like glycogen (6, 7) and appears to arise from undefined defect or misregulation in the glycogen synthesis and degradation in which the Laforin could be involved.

Because Laforin is expressed ubiquitously, it is likely to play a general role in cellular physiology. We have recently demonstrated that insertion-mediated mutagenesis of Epm2a leads to rapid onset of lymphoma in immune compromised mice (8). Importantly, we found that Laforin is an important regulator of Wnt signaling in cancer development (8). Moreover, we and others have reported that Laforin is a phosphatase that regulates the critical Ser9 phosphorylation under a variety of physiological context (9). Given the important function of GSK3β in signal transduction, understanding the biochemical mechanism of Laforin function may provide important mechanistic insights on a number of important signal transduction pathways, cellular physiology, and carcinogenesis.

The human and mouse EPM2A have 94% homology at the amino acid levels (3) and have four exons. Exon 1 encodes a carbohydrate binding domain (CBD), and the range from the end of exon 2 to the partial exon 4 encodes a DSP domain (3, 10). Most of the known missense mutations found in the LD locate in the two domains (11, 12). The W32D mutation in the CBD losses its binding to glycogen in vitro (10). Further studies revealed that many single substitution mutants not only in CBD but also in the DSPD are unable to bind to glycogen, and that these mutants incapable of binding to glycogen also lose or attenuate their phosphatase activity (13). When expressed in yeast, Laforin interacted with itself and the Laforin mutations that inactivated its phosphatase activity prevented this interaction (13). These data raised an intriguing possibility that Laforin function may depend on its dimerization. This requirement would offer plausible explanation for why mutations outside the phosphatase domain may have significant effect on its phosphatase activity (10, 13). Here we present evidence that dimerization is critical for phosphatase activity, regulation of GSK3β phosphorylation, and Wnt signaling. Our data suggest a new dimension in explaining the pathogenesis of LD.

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3 The abbreviations used are: LD, Lafora Disease; DSP, dual specificity protein phosphatase; CBD, carbohydrate binding domain; IP, immunoprecipitation; GST, glutathione S-transferase; WT, wild type; p-NPP, para-nitrophenylphosphate; CBP, calmodulin-binding peptide; DTT, dithiothreitol.
**EXPERIMENTAL PROCEDURES**

*Plasmids*—Full-length cDNA of Epm2a were amplified by reverse transcription PCR using high fidelity Taq enzyme (Invitrogen) from normal human tonsil and mouse spleen and cloned into vectors of pcDNA3.1-Myc/His and pcDNA4-V5/His (Invitrogen) at restriction enzyme sites of HindIII/BamHI to get fusion proteins with Myc or V5 tag fused in the C terminus of Laforin. Cloning Epm2a cDNA into pCTAP vector (Stratagene) generated a fusion protein with streptavidin-binding peptide and calmodulin-binding peptide named CBP tag fused in the C terminus of Laforin. All mutants were made by site-directed mutagenesis using the template of wild-type Epm2a. Deleted mutants of Epm2a were amplified by PCR using variant splicing transcripts (found by us) as templates and cloned sites into pcDNA vectors. Prokaryote fusion protein expression vector pGEX-6.1-P (Promega) and pET-21b (Novagen) were used to make expression plasmids of GST-Laforin and GST-C265S and Laforin-His and C265S-His, respectively. All expression plasmids were confirmed by sequencing.

*Transfection*—Human embryonic kidney (HEK293) cells were transiently transfected or cotransfected with different expression plasmids premixed with lipofectin-2000 and cultured for 24 h in Opti-MEM medium containing 10% fetal bovine serum. To achieve a similar level of protein expression between wild-type to mutant Laforin, we used 3-fold less wild type and C265 mutant plasmids than all other mutants studied.

*Immunoprecipitation and Western Blot*—Transfected HEK293 cells were lysed by a lysis buffer containing 1% Triton X-100 and cocktails of protease inhibitors and phosphatase inhibitor (Sigma) at 1:100 dilutions. The lysates were pre-cleared with protein G beads (Amersham Biosciences) for 1 h and were incubated with monoclonal antibodies specific for either Myc or V5 tags under constant rotation overnight at 4 °C. The protein G beads were then added and incubated for an additional 2-h rotation at 4 °C. The beads were washed with lysis buffer three times, and phosphatase assay buffer once if the IP complex is used for phosphatase assay. The IP complex was dissociated from protein G beads by SDS-PAGE loading buffer and separated in a 10% SDS-PAGE gel. Thereafter, the proteins were transferred into nitrocellulose membrane and probed with monoclonal antibodies. The primary antibodies used in Western blot were: anti-Myc or anti-V5 (Invitrogen); anti-p-GSK3β, anti-p-GSK3β (Ser9), β-catenin (Cell Signaling); SP1 (Santa Cruz Biotechnology); anti-mouse monoclonal His (Novagen), anti-goat polyclonal GST (Amersham Biosciences), and anti-rabbit polyclonal Laforin made by Genemed Synthesis, Inc (San Francisco, CA). The second antibodies used were: anti-mouse and rabbit horseradish peroxidase (Cell Signaling), anti- goat-horseradish peroxidase (Amersham Biosciences).

*Protein Purification*—Prokaryote GST-and His-fused proteins were expressed in *Escherichia coli* BL21 and BL21(DE3)P1ys and purified by GST resin (Sigma) or nickel beads (Novagen) as described previously (10).

*Phosphatase Activity Assay*—Phosphatase activity of Laforin was measured using para-nitrophenylphosphate (p-NPP) as described before (13). Briefly, purified recombinant protein (10 ng to 1 μg or 30 μl of 1:500 diluted slurry of fusion Laforin immobilized on beads) was incubated with 10 μM p-NPP at 37 °C for 30 min in a total volume of 100 μl of buffer (50 mM Hepes pH 6.0, 50 mM NaCl, 5 mM EDTA, and 50 mM β-mercaptoethanol). The reaction supernatant was terminated by 10 μl of 1 M NaOH. The converted products were measured by absorbance at 410 nm, and a molar extinction coefficient of 1.78 × 10^4 was used to calculate the phosphatase activity.

When bead-bound enzymes were used, the amounts of enzymes in each reaction was determined by SDS-PAGE after the proteins were released by boiling with reducing SDS buffer, using BSA as standard. The gels were stained with Coomassie Blue and the intensity of scanned bands of targets, and bovine serum albumin was determined using SigmaPlot.

*Sucrose Gradient Ultracentrifugation*—Cell lysates were fractionated by sucrose gradient ultracentrifugation as described (14). Wild type and W32D mutant V5 were transiently transfected into HEK293. 24-h later, the cells were lysed, and the lysates were laid on the top of a 5–20% sucrose gradient and separated by ultracentrifugation using SW50.1 and at a speed of 35,000 rpm for 20 h. The 200-μl fractions were collected, and the amounts and molecular weight of the Laforin-V5 is measured by Western blot using anti-V5 mAb.

*Gel Filtration*—Lysates from 2 liters of BL21 culture were applied to a nickel column. The Laforin-His proteins were eluted with imidazole (250 mM). The eluted proteins were separated by a Sephacryl S-300 High Resolution gel filtration column (Amersham Biosciences) equilibrated with running buffer (10 mM Tris-HCl and 150 mM NaCl) at a flow rate of 0.5 ml/min and collected in 1.5-ml fractions. Protein contents were monitored by the absorbance of 280 nm. Molecular weight (MW) markers from Amersham Biosciences were used to determine MW distribution in the eluate. The fractions from each peak were collected and the proteins were re-isolated using the His beads and suspended in the phosphatase buffer to measure the phosphatase activity.

**RESULTS**

Laforin Forms SDS-resistant Dimers in Mammalian Cells—Our previous studies indicated that Laforin is expressed at very low levels in hematopoietic malignant cells (8), although some non-hematopoietic tumor cells, such as breast cancer cell line 4T1, express significant levels. Interestingly, under the non-reducing SDS-PAGE that involved no DTT and no boiling of samples, the major band that reacted with anti-Laforin antibody is ~80 kDa, although a smaller amount of the 57-kDa species is also found. With reduction and boiling prior to SDS-PAGE, a major portion of the 80-kDa band was converted into a new 40-kDa band (Fig. 1A). Whereas the abundance of the 57-kDa band was also increased with reduction, this band was not found in most of cell lines tested by the anti-Laforin antibody (data not shown), we therefore consider that the 57-kDa band to be either post-translationally modified form of the Laforin or nonspecific. The conversion from 80 to 40 kDa by reduction has been observed in another mouse mammary tumor cell line TSA (data not shown). This result raised the intriguing possibility that the 80-kDa band may be a dimeric form of the 40-kDa band. To confirm this possibility, we expressed recombinant Laforin with V5 tag at its C terminus in

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**Dimerization and Phosphatase Activity of Laforin**

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Dimerization and Phosphatase Activity of Laforin

293 cells. The expressed proteins were immunoprecipitated with anti-V5 antibody. The immunoprecipitates were treated with DTT and SDS and boiled prior to Western blot. As shown in Fig. 1B, even after these treatments, a significant amount of Laforin-V5 dimer was detected.

An aliquot of immunoprecipitates was used to measure the phosphatase activity using the generic phosphatase substrate, p-NPP. The Laforin-V5 precipitates had very high phosphatase activity. As expected, mutation at the phosphate active site C265S completely abrogated the phosphatase activity. Because this mutant still formed dimer under the same condition, it is likely that Laforin dimerization does not require its phosphatase activity.

To further substantiate the notion that Laforin form dimers in mammalian cells, we fused mouse (mL) or human (hL) Laforin with different tags in the C terminus. Two cDNAs were cotransfected into the 293 cells. The cell lysates from the transfectants were precipitated with anti-V5 antibodies. The immunoprecipitates were further analyzed by a reducing SDS-PAGE after treatment with DTT and boiling. Western blot with antibodies specific for different tags provided two lines of evidence to support dimerization of Laforin. First, in addition to the monomeric, dimeric forms of hL and mL could be observed at high quantity regardless of the combination used. The slight shifts in molecular weight were consistent with the notion that mouse and human Laforin can form both homodimers and/or heterodimers. Second, the anti-V5 antibody pulled-down substantial amounts of CBP-tagged (Fig. 2, middle panel) and Myc-tagged (right panel) Laforin of both mouse and human origins. Taken together, data presented in this section indicate that Laforin can form dimers that are substantially resistant to boiling under reducing condition in the presence of SDS.

Structural Functional Analysis Revealed a Critical Role of Dimerization in Phosphatase Activity—We took two approaches to determine whether dimerization of Laforin is essential. First, we deleted different portions of Laforin and determined the regions required for homodimerization. All deletion mutants were fused with either Myc or V5 tag at their C terminus. The same mutants with different tags were cotransfected into the 293 cells. The cell lysates were precipitated with anti-V5 and blotted with anti-Myc to reveal homodimerization. As shown in Fig. 3A, 5 deletion mutants were produced, each losing part of the amino acids encoded by exons 1–3. While full-length Laforin homodimerized readily, deletion of amino acids encoded by the entire exon 1 or part of it (deletion from amino acid residues 13–65) completely abrogated homodimerization (Fig. 3B). Thus, exon 1-encoded amino acids are critical for homodimerization. Although deletion of exons 2 and 3 or a large fragment spanning residues encoded by part of exon 2, the entire exon 3 and a small portion of exon 4 (named as dL2p3) did not completely abrogate homodimerization, a substantial reduction was also observed (Fig. 3B). It is therefore likely that multiple regions in Laforin are involved in homodimerization. Consistent to the lost dimerization, none of the deleted forms had significant phosphatase activities (Fig. 3C).

A number of natural mutations have been described in the EPM2a gene (3, 12). Because these mutations abrogated the physiological function of Laforin, we tested whether these mutations inactivated dimerization and/or phosphatase activity by introducing the individual mutation into the Epm2a cDNA (Fig. 4A). The wild-type and mutant Laforins were fused with Myc or V5 tag at their C terminus, and the Myc and V5
Dimerization and Phosphatase Activity of Laforin

**FIGURE 3.** Deletional analysis revealed involvement of a large area of Laforin in its dimerization and phosphatase activity. A, diagram of deletion mutants generated. Regions of CBD and DSP are illustrated with *unfilled boxes* on the top. Relative sizes and positions of the deletion mutants were shown with *filled bars*. B, WT and mutant Laforin in A were tagged at the C termini with either V5 or Myc. cDNA with the two different tags were transfected into HEK293 cells. The lysates were precipitated with anti-V5 antibodies, and the amounts of immunoprecipitated protein were determined by blotting with anti-V5 antibodies. The amounts of V5-tagged protein in the lyses were determined by blotting with anti-Myc or anti-V5 antibodies. C, phosphatase activity of deletional mutants of Laforin. Each V5-tagged mutant was transfected into HEK293 cells and lysed for immunoprecipitation using anti-V5 antibody. p-NPP was used as substrate.

plasmids of each were co-transfected into the 293 cells, and the association between Myc and V5 of each were detected by IP-Western blot. As shown in Fig. 4B, despite comparable expression, substantial reductions of dimerization were observed in all three exon 1 mutants, and the mutants in exons 3 and 4. Two mutants of F83L in exon 1 and Q292L in exon 4 showed the lowest ability to dimerize. Quantitative analysis demonstrated that mutants F83L and Q292L reduced dimerization by more than 90% (data not shown), while other mutants tested reduced dimerization by 80%. The phosphatase dead mutant (C265S) retained its ability to dimerize, which supports our contention that dimerization does not require phosphatase activity. Importantly, for all other mutants, the impact of the mutation on Laforin dimerization was matched with that on phosphatase activity (Fig. 4C). These data are consistent with the notion that dimerization may contribute to its phosphatase activity.

**Differential Sedimentation of Wild-type and Mutant Laforin**—To determine whether a mutation in the CBD domain affects molecular association of Laforin, we lysed 293 cells that were transfected with either the wild-type or the W32D mutant of Epm2a with 1% Triton X-100. The lysates were then overlaid into a sucrose gradient of 5–20%, and separated by a 24-hour ultracentrifugation. The fractions collected were analyzed by reducing SDS-PAGE followed by Western blot to determine sedimentation profiles of Laforin. As shown in Fig. 5, the major-detectable phosphatase activity of the mutant (Fig. 4C).

Essentially All Enzymatic Activity of the Recombinant Laforin Produced in Bacteria Is Attributable to Dimers—Consistent with the published data (10), we found that the recombinant soluble Laforin produced in bacteria had detectable but low phosphatase activity. This was due to difficulties in eluting dimers from the nickel columns (supplemental Fig. S1) and that high concentration of imidazole used for elution irreversibly inhibited Laforin activity (data not shown). Moreover, we observed that tagging Laforin at the N terminus eliminated the dimer formation and drastically reduced phosphatase activity (Fig. S1). The position of the Tag in different studies may explain the difference in both activity and specificity of Laforin reported by different groups (8, 9, 15).

To determine whether the phosphatase activity of Laforin-His in the solution is contributed by the dimers, the recombinant proteins were separated by gel filtration. As shown in Fig. 6A, two species of His-tagged Laforin proteins were detected, with their sizes consistent with monomeric (major) and dimeric (minor) Laforin. The fractions within each peak were then pooled and re-precipitated with nickel beads and assayed for phosphatase activity. As shown in Fig. 6B, only dimeric forms were active. These data demonstrated that dimerization is essential for the phosphatase activity of recombinant Laforin expressed in bacteria.
Requirement for Dimerization for Laforin-mediated Regulation of GSK3\(\beta\) Phosphorylation and Wnt Signaling—We have recently demonstrated that Laforin is an important regulator of GSK3\(\beta\) activity as it specifically dephosphorylates GSK3\(\beta\) at the critical Ser\(^9\) residue. To determine whether dimerization is required for this important cellular function, we compared wild-type and mutant Laforin for their ability to regulate GSK3\(\beta\) phosphorylation at the Ser\(^9\) residue induced by insulin. As shown in Fig. 7A, treatment of insulin induced a robust phosphorylation of GSK3\(\beta\) at the Ser\(^9\) position. This induction was completely abrogated by wild-type Laforin. Interestingly, all of the mutants tested lost this function regardless of whether they reside within the phosphatase domain. Since all of the naturally occurred mutations that impaired dimerization also lost the ability to regulate GSK3\(\beta\) phosphorylation at Ser\(^9\), we conclude that dimerization is critical for regulation of GSK3\(\beta\) by Laforin.

We have also established that Laforin regulates Wnt signaling during cancer development. We therefore evaluated whether the function of Laforin in Wnt signaling also requires dimerization by comparing the activity of the wild type and the mutants. As shown in Fig. 7B, transfection of wild-type Laforin prevented Wnt-induced \(\beta\)-catenin translocation in the nuclei. In contrast, the mutants that failed to form dimers did not suppress Wnt signaling. Thus, dimerization is likely important for Laforin-mediated regulation of Wnt signaling.

**DISCUSSION**

Laforin is a dual-specific phosphatase, which is mutated in patients with epilepsy of progressive myoclonus. The mutation not only occurred in the DSPD, but also in the CBD. Recent work by Wang et al. (10) established that the domain is involved in glycogen binding, although interaction with other types of carbohydrate has also been suggested. Interestingly, although reports from different groups vary in the extent of impact (10, 13), all studies demonstrate a significant effect of the CBD mutations in the phosphatase activity using generic phosphatase substrates. Here we showed that Laforin naturally form stable dimers that are at least partially resistant to SDS treatment under boiling and reducing conditions. Moreover, structural functional analyses of Laforin support a critical role of dimerization in its phosphatase activity, regulation of GSK3\(\beta\) activity, and Wnt signaling, although dimerization does not require phosphatase activity. Our conclusion is based on several lines of evidence.

First, Laforin found in cultured cell lines were SDS-resistant dimers. While reduction and boiling converted some of the dimers into monomers, this conversion is never complete in our experience. Thus, the force responsible for dimerization was at least in part independent of the disulfate bond. Using...
Laforin tagged at its C terminus, we observed SDS-resistant dimers of Laforin formed in both bacterial and mammalian cells. The existence of the Laforin dimer in untransfected cells reveals that dimerization is not an artifact of overexpression.

Second, deletional analysis revealed that a large area of the Laforin protein is involved in dimerization. Deletion of the CBD domain completely abrogated dimerization. An interesting issue is whether the binding to glycogen is involved in dimerization. We believe this is less likely as recombinant protein produced in bacteria, which lack glycogen, also dimerize. In addition, deletion of exon 2 and/or 3 encoded region, which has not been implicated in glycogen binding, also caused a substantial reduction in Laforin dimerization. The involvement of a large area is further substantiated by the fact that naturally occurring mutations in exons 1 and 4 can cause substantial reduction of Laforin dimerization. The involvement of large area may explain the stability of the dimers.

Third, dimerization is critical for the enzymatic activity of Laforin. We have demonstrated that deletions or naturally occurring mutations that abrogate dimerization of Laforin inactivate the phosphatase. Likewise, GST-tagged Laforin at N terminus also prevented dimerization and abrogated the phosphatase activity (supplemental Fig. S1). Furthermore, gel filtration confirmed that the dimer, but not the monomer, of recombinant protein produced from bacteria has phosphatase activity. Sucrose gradient ultracentrifugation defined that the impaired dimerization of the W32D mutant at exon 1 corresponds to the reduction of its phosphatase activity. However, it should be noted that enzymatic activity is not required for dimerization, as C265S mutated at the critical residue for enzymatic activity completely inactivated the phosphatase but did not affect dimerization.

Fourth, dimerization of Laforin is not only required for its generic phosphatase activity, but also essential for its regulation of GSK3β and Wnt signaling, two important functions recently attributed to Laforin (8, 9). Thus, while WT Laforin suppressed insulin-induced GSK3β phosphorylation at the Ser9 position, all mutants that lost the ability to form dimers were unable to dephosphorylate GSK3β. Likewise, three dimer-
Dimerization and Phosphatase Activity of Laforin

ization-defective mutants tested also failed to suppress Wnt-induced accumulation of β-catenin in the nuclei. These data further strengthen the importance of dimerization in Laforin function in vivo. While the structural requirements for GSK3β dephosphorylation at Ser⁹ and suppression of Wnt signaling are similar, it is premature to conclude that Laforin regulate Wnt signaling by modulating GSK3β phosphorylation at the Ser⁹ position. Recent studies indicated that mice with mutant GSK3β Ser⁹→Ala GSK3α Ser²¹→Ala mutations have normal development (16), which suggests that Wnt signaling is not regulated by phosphorylation at these residues. Moreover, MEF prepared from the mutant mice had normal response to Wnt in vitro. This apparent inconsistency can be reconciled either by postulating other potential phosphorylation sites on GSK that regulate Wnt signaling or alternatively by suggesting that Laforin regulates Wnt signaling by GSK-independent mechanisms. Regardless of what the ultimate mechanisms are, the fact that naturally occurring mutation of Laforin abrogates their ability to regulate GSK and Wnt signaling suggested a new dimension in explaining the pathogenesis of Lafora’s diseases.

Taken together, our data provide a clear-cut example on the significance of dimerization in function of an important protein phosphatase. Our data extend the growing body of evidence on the role for dimerization in phosphatase activity and regulation (17–21). Because essentially all of the naturally occurred mutations abrogate dimerization of Laforin, dimerization defect may be an essential pathway for the molecular defects in the Lafora diseases.

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