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Modulation of functional properties of laforin phosphatase by alternative splicing reveals a novel mechanism for the *EPM2A* gene in Lafora progressive myoclonus epilepsy

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The *EPM2A* gene, encoding the dual-phosphatase laforin, is mutated in a fatal form of progressive myoclonus epilepsy known as Lafora disease (LD). The *EPM2A* gene, by differential splicing of its transcripts, is known to encode two laforin isoforms having distinct carboxyl termini; a major isoform localized in the cytoplasm (laf331), and a minor isoform that is targeted to the nucleus as well (laf317). We show here that the two laforin isoforms interact with each other and form homo and heterodimers. The homodimer of laf331 display robust phosphatase activity, whereas the laf317 homodimer and the laf331–laf317 heterodimer lack phosphatase activity. Laf331 binds to glycogen only as a monomeric form. Laf317, on the other hand, was unable to bind to glycogen as a homodimer or as a heterodimer. Similar to laf331, laf317 interacts with and functions as a substrate for the malin ubiquitin ligase—a product of another gene defective in LD. Malin, however, shows higher affinity towards laf331 when compared with laf317. We have also tested the effect of LD-associated mutations, whose effects are restricted to the laf331 isoform, on laf331–laf317 interaction. Two such mutations are known and both abolish the interactions between laf317 and laf331 and their heterodimerization, but not the homodimerization property of laf331. Thus, laf317 could function as a dominant-negative regulator of laf331, and laf331-specific mutations might affect laf317 functions as well. Thus, our findings reveal a novel mechanism for the *EPM2A* gene function, regulated by alternative splicing, in normal as well as disease conditions.

INTRODUCTION

The Lafora-type progressive myoclonus epilepsy, also known as Lafora disease (LD), is an autosomal form of genetic disorder characterized by the presence of Lafora polyglucosan bodies in the affected tissues, including the neurons (1). The symptoms of LD include myoclonic and absence seizures, drop attacks, ataxia and a quickly developing, progressive and severe dementia (2). LD is caused by at least three genes, of which two have been identified and characterized. These are *EPM2A* and *NHLRC1* (reviewed in 1). The *NHLRC1* gene encodes an E3 ubiquitin ligase and LD-associated mutations affect its ligase activity and/or the subcellular localization (3–5). The *EPM2A* gene product laforin is a dual-specificity protein phosphatase (6,7) and LD-associated mutations are known to affect its phosphatase activity, glycogen binding affinity and subcellular localization (7–12). Laforin and malin physically interact, co-localize and thought to work together in cellular cascades that are critical for neuronal functions (1,3–5). Consistent with this view, malin was shown to ubiquitinate protein targeting to glycogen (PTG), a regulatory subunit of protein phosphatase-1, which enhances glycogen accumulation in a laforin-dependent manner (13,14). Thus, loss of functional laforin or malin would result in increased PTG levels, leading to the genesis of polyglucosan inclusions in the neurons (13–15).

The *EPM2A* transcript in human is known to undergo alternative splicing resulting in the production of at least two laforin isoforms with distinct carboxyl termini, one localizing in the cytoplasm and the other in the nucleus (16).

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Intriguingly, two mutations in the *EPM2A* gene that specifically target the cytoplasmic isoform are known in LD (17,18). It has been suggested, therefore, that the nuclear isoform is not relevant to the LD pathology (17). Since the cytoplasmic laforin is known to form homodimers and the dimerization is required for the enzymatic activity (12), we have recently proposed that the two isoforms of laforin could form heterodimers with distinct functional properties (18). In this report, we demonstrate the physical interaction between laforin isoforms and with malin, and show that the nuclear isoform exhibits a dominant-negative activity over the cytoplasmic form.

RESULTS

Laforin isoforms physically interact with each other and form homo- and heterodimers

Differential splicing in the *EPM2A* gene transcript lead to the production of two distinct laforin isoforms with distinct carboxyl-termini: a 331 amino acid long major cytoplasmic isoform (hereafter identified as laf331) (7), and a 317 residue long minor nuclear isoform of laforin (laf317) (16) (see Fig. 1A and B). Because laf331 is known to form functional dimers (12), we wanted to check whether laf317 would also form dimer with itself (homodimer) and/or with the laf331 (heterodimer). For this, we created laf331 and laf317 expression constructs having the amino-terminal FLAG tag or the carboxyl-terminal Myc/His tag (see Materials and Methods). Transient expression of these constructs showed distinct localization patterns for the two isoforms as demonstrated earlier (16); laf331 was predominantly cytoplasmic in localization, whereas laf317 was localized both in nucleus and cytoplasm (Fig. 1C). We next established that both laf317 and laf331 form dimers *in vivo*. For this, we transiently expressed laf317 and laf331 in COS-7 cells, either treated or not treated with a chemical cross-linker, disuccinimidyl suberate (DSS), and then analyzed by immunoblotting. As shown in Figure 2A, in addition to the monomeric band, both isoforms formed a prominent band, at a higher molecular weight (~80 kDa), representing the homodimers in the cell lysates treated with the cross-linker. The dimeric form was not detected when the cells were not treated with DSS, suggesting that they were specific signals. To obtain independent evidence for the observed dimerization, we have used expression constructs that encode proteins with distinct epitope-tags and used them for the pull-down experiments. For this, COS-7 cells were co-transfected with expression constructs encoding Myc/His-tagged laf-331 and FLAG-tagged laf331, Myc/His-tagged laf331 and FLAG-tagged laf317, or Myc/His-tagged laf317 and FLAG-tagged laf317, and the cell lysates were processed for pull downs using Nickel resins followed by immunoblotting (Fig. 2B). Indeed, co-precipitation was observed in lysates prepared from cells that had expressed the same isoform with two distinct tags, or both isoforms each with a unique tag, confirming that isoforms laf331 and laf317 physically interact with themselves and with each other, thereby suggesting the presence of homo- and heterodimeric forms of laforin phosphatase (Fig. 2B). Control pull-down with Nickel resin in a lysate that had the FLAG-tagged protein but not the Myc-tagged protein confirmed the specificity of these assays (Fig. 2B).

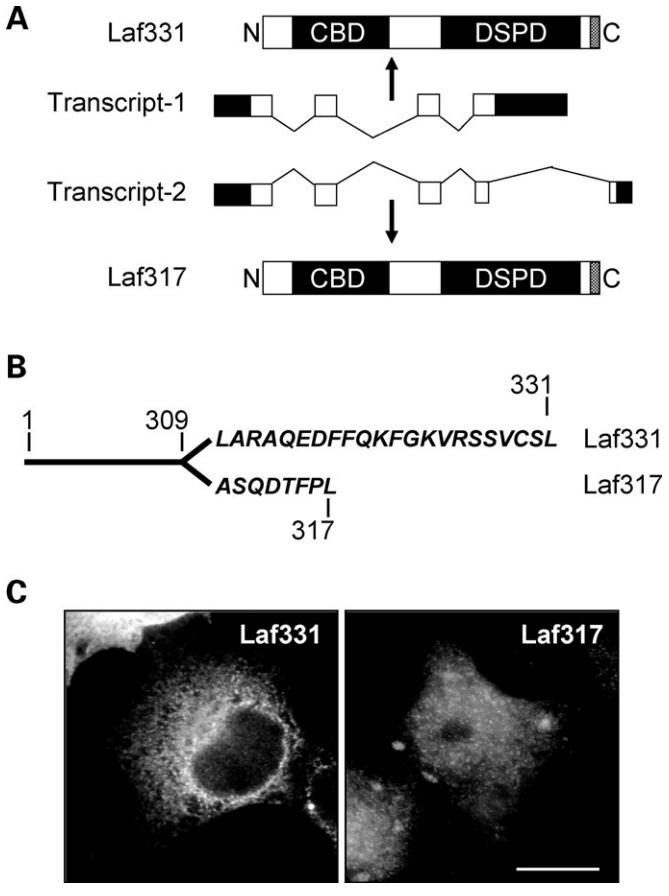

Figure 1. Domain organization and subcellular localization patterns for laforin isoforms. (**A**) Schematic diagram showing the two differentially spliced transcripts of the *EPM2A* gene, and the encoded proteins. For the transcripts, boxes denote the exons, and the filled blocks are the 5′- and 3′-untranslated regions (UTRs). Transcript-1 encodes a 331 amino acid long protein, named laf331, and transcript-2 encodes a 317 amino acid long protein, named laf317. The relative positions of the two domains of laforin, carbohydrate-binding domains (CBD) and dual-specificity phosphatase domain (DSPD), are also shown. The two domains are present in both isoforms. The unique sequence at the carboxyl-terminal of the two isoforms is identified by the 'hatched' box. (**B**) A schematic diagram showing the carboxyl terminal of laf331 and laf317 and the amino acid sequence of unique regions. (**C**) Representative images of COS-7 cells showing the localization pattern of laf331 and laf317. For this, COS-7 cells were transiently transfected with expression constructs that code for Myc-tagged laf331 or laf317 and processed for immunofluorescence staining using anti-myc antibody. Scale bar, 10 μm.

To further validate the heterodimerization property of these isoforms, laf331 and laf317 expression constructs with Myc/His-tag and FLAG tag, respectively, were co-expressed in COS-7 cells and the poly-histidine-tagged laf331 was pulled-down using Ni-affinity resins (see Materials and Methods). Upon DSS-mediated chemical cross linking, isoform laf317, which should have been pulled-down by laf331, showed a higher molecular weight corresponding to the heterodimers (~80 kDa) in addition to a monomeric band (Fig. 2C). This dimeric form was detected both by anti-Myc and anti-FLAG antibodies, thus establishing the presence of heterodimers (Fig. 2C). Laf317 and laf331 showed significant overlap for

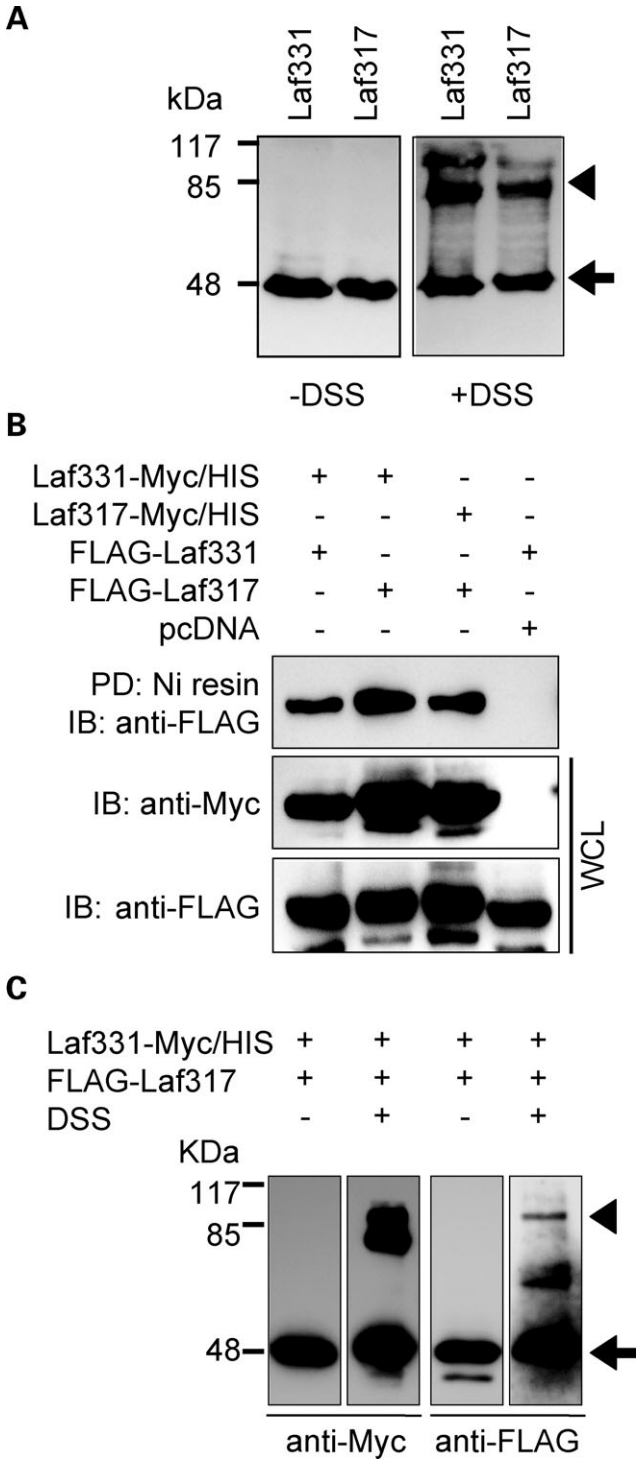

Figure 2. The two isoforms of laforin interact with each other and form homodimers as well as heterodimers. (**A**) Laforin isoforms, laf331 and laf317, over-expressed in COS-7 cells were treated (+) or not treated (−) with the cross-linker DSS, resolved in SDS–PAGE and detected by immunoblotting. Anti-laforin antibody detected a band at around 80 kDa, representing the dimeric form for both laf331 and laf317 (identified by an arrow head) in the samples that were treated with DSS. The monomeric form (∼45 kDa; identified by an arrow) was present both in DSS-treated and -untreated samples. (**B**) COS-7 cells were transiently co-transfected with expression constructs as indicated, and processed for pull down (PD) using nickel affinity resin and by immunoblot (IB) analysis using anti-FLAG antibody. Cells that were expressing only the FLAG-tagged protein were processed in parallel as negative control (last lane). The PD products and the whole cell lysates (WCL) were used for the IB detection. (**C**) To establish that laf331–laf317 heterodimer indeed forms, lysates from COS-7 cells that were expressing Myc/His-tagged laf331 and FLAG-tagged laf317 were processed for affinity purification using Ni-resin. The purified protein samples were either treated or not treated with DSS, resolved in the same gel, transferred to the membrane and the same blot was probed with anti-Myc, and after stripping, with anti-FLAG antibodies. The higher molecular weight band (dimeric form identified by an arrowhead) detected by the anti-Myc antibody was also detected by the anti-FLAG antibody, thus establishing the presence of laf331–laf317 dimeric complex. The monomeric forms are identified by an arrow.

fluorescence staining pattern when co-expressed in COS-7 cells, and the subcellular localization pattern of laf317 was not affected when co-expressed with laf331 (Fig. 3E–G).

Laf317 is an inactive phosphatase that competes with laf331 for the substrate and regulates the activity of laf331 by forming heterodimers

We and others have shown earlier that laf331 is a dual-specificity phosphatase (7,9). In order to test whether laf317 exhibits phosphatase activity or not, COS-7 cells were transiently transfected with the construct that codes for Myc/His-tagged laf317 and purified the proteins using Ni-affinity resins. Affinity purified products from COS-7 cells that express Myc/His-tagged laf331 or an empty vector were used as positive and negative controls, respectively. The resin-bound proteins were used for the phosphatase assay with the chromogenic substrate, para-nitrophenyl phosphate (pNPP) (Fig. 4A). As demonstrated earlier (7), laf331 showed robust phosphatase activity with pNPP; however, laf317 did not show appreciable levels of activity with pNPP under identical assay conditions, and the absorbance was similar to the assay done with resins derived from the vector transfected cells, suggesting that laf317 could perhaps be an inactive phosphatase (Fig. 4A).

Since laf317 interacted with laf331, we next checked whether the laf317–laf331 heterodimeric form would be an enzymatically active complex or not. For this, we transiently over-expressed Myc/His-tagged or FLAG-tagged laf317 and laf331—either together or separately as indicated in Figure 3B, and purified them using affinity resins. The resin bound proteins were tested for the phosphatase activity with pNPP. As a control, we have used a phosphatase inactive mutant of laf331, C266S (9,19), to establish the specificity of the assay conditions (Fig. 4B). These experiments were done in triplicate to account for any variations in the experimental conditions. The presence of homo- and heterodimeric forms of laforin isoforms in the pulled down products was confirmed by immunoblotting with anti-Myc and anti-FLAG antibodies. As shown in Figure 4B, the laf331 homodimer, as expected, has shown robust phosphatase activity. However, the homodimeric forms of laf331 C266S mutant or laf317 did not show any appreciable level of activity. Furthermore, the activity of laf331-Myc/His, as heterodimer with FLAG-laf317, was reduced to nearly 60% to that of laf331 homodimer (Fig. 4B). Since laf317 was pulled-down using His-tagged laf331, the observed difference in the activity could be either

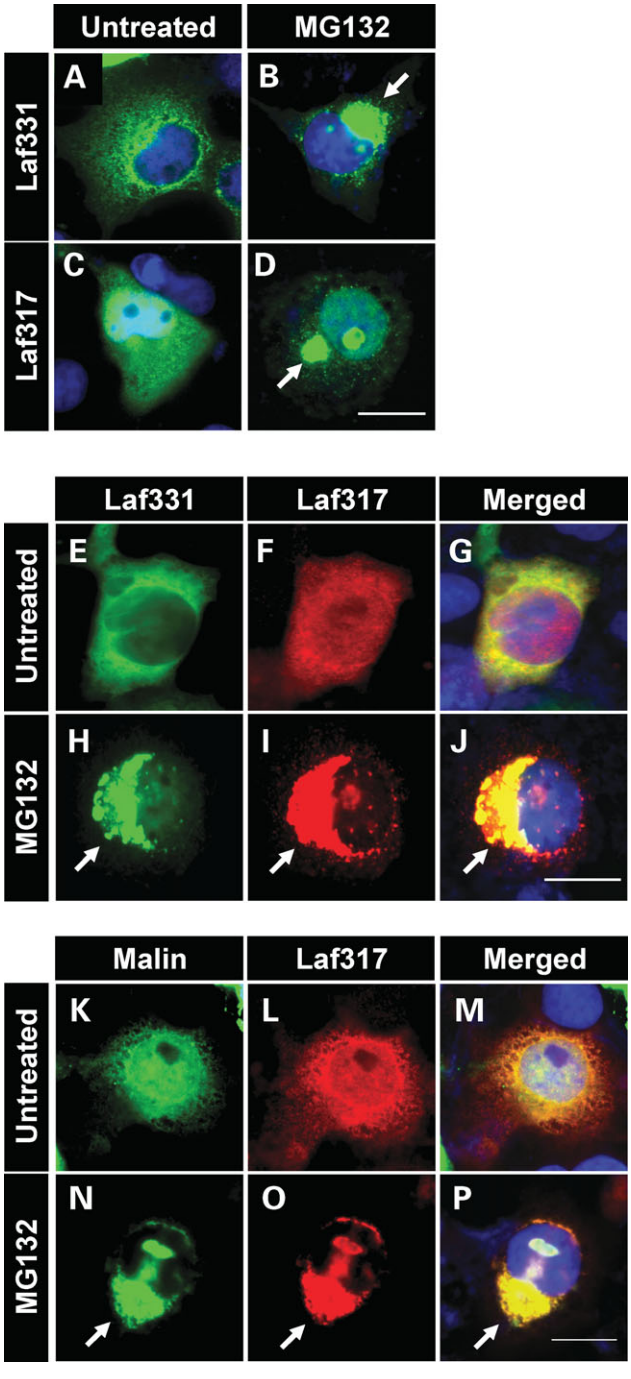

Figure 3. Laf317 co-localize with laf331 and malin, and form aggresome upon proteasomal blockade. Laforin isoforms laf317 and laf331 were transiently expressed in COS-7, either separately (**A**–**D**) or together (**E**–**J**), and were either treated (B,D, H, I and J)) or untreated (A, C, E, F and G) with MG132 for 12 h. Twenty-four hours post-transfection, cells were processed for indirect immunofluorescence staining using appropriate antibodies. Laf317 was also co-expressed with malin to see if they both co-localize (**K**–**P**). For this, cells were treated either not treated (K, L and M) or treated (N, O and P) with MG132 and processed for indirect immunofluorescence staining. For all the experiments, cells were counterstained with DAPI to reveal nucleus. Arrows indicate aggresomal structures. Scale bar, 10 μm.

due to the laf331–laf317 heterodimers, or due to the presence of lower levels of laf331 homodimers in the pulled down products. We reasoned that the latter possibility is likely, because, in our pull-down assays, laf331 had consistently showed higher affinity to itself when compared with laf317 (see Fig. 4B). We have, therefore, used laf317-Myc/His for the pull-down of laf331-FLAG and checked the activity of this complex with pNPP (Fig. 4B). Since laf317 dimer is an inactive phosphatase, pNPP activity if detected for this pull-down product should come from the laf331–laf317 heterodimers. While laf317–laf331 heterodimers were indeed present in the pull-down products, this complex did not show detectable levels of phosphatase activity (Fig. 4B). This observation suggests that the laf331–laf317 heterodimer is an inactive complex. In these assays, we believe that the heterodimers were stable and did not dissociate to form homodimers because, laf331-FLAG that were pulled with the His-tagged laf317 or the laf331 mutant C266S should have shown some activity when assayed with pNPP if it were the case. We therefore next examined whether laf317 would compete with laf331 for the substrate *in vitro*. To test this possibility, we mixed increasing amounts of purified laf317 dimers with constant amount of laf331 dimers and checked for the phosphatase activity with pNPP as the substrate. As shown in Fig. 4C, increasing concentrations of laf317 progressively diminished the phosphatase activity displayed by laf331 in a dose-dependent manner, suggesting that laf317 could possibly compete for the substrate by binding to, and/or preventing its dephosphorylation by laf331.

Laf317 does not bind to glycogen *in vitro*

We and others have shown that the purified laf331 binds to glycogen *in vitro* (11,20). Laf331 also binds to Lafora polyglucosan bodies (11,21), and has been shown to dephosphorylate carbohydrate complexes (22). We were, therefore, curious to check whether laf317 would bind to glycogen or not. For this, Myc/His-tagged laf317 and laf331 were expressed separately in COS-7 cells, purified using Ni-resins and tested for their affinity towards glycogen particles *in vitro* (see Fig. 5A). As demonstrated earlier, laf331 sedimented with glycogen in the pellet fraction, establishing its strong affinity towards the glycogen-like complexes (Fig. 5A). Laf317, on the other hand, was found in the supernatant fraction, suggesting its lack of affinity towards glycogen particles (Fig. 5A). Because laf331 forms dimer, and that the dimeric form is active as a phosphatase, we have also tested whether the observed glycogen affinity for laf331 is dependent upon its dimerization. For this, we co-expressed two constructs that code for laf331-Myc/His and FLAG-laf331 respectively, purified the dimers using Ni-affinity resins, and did the glycogen binding assay (Fig. 5B). While Myc-tagged laf331 was seen both in the glycogen pellet and supernatant fractions, FLAG-tagged laf331 was seen only in the supernatant fraction. Since the FLAG-tagged laf331 is expected to be present only as a dimer in our pulled-down products, the absence of any signal for FLAG epitope in the pellet fraction suggest that homodimers of laf331 do not bind to glycogen *in vitro* and the observed affinity could perhaps be restricted to the monomeric form. Since laf331 and laf317 form heterodimer, we next checked whether the heterodimer would bind to glycogen or not. For this, the purified heterodimers were incubated with glycogen and checked for their affinity. As shown in

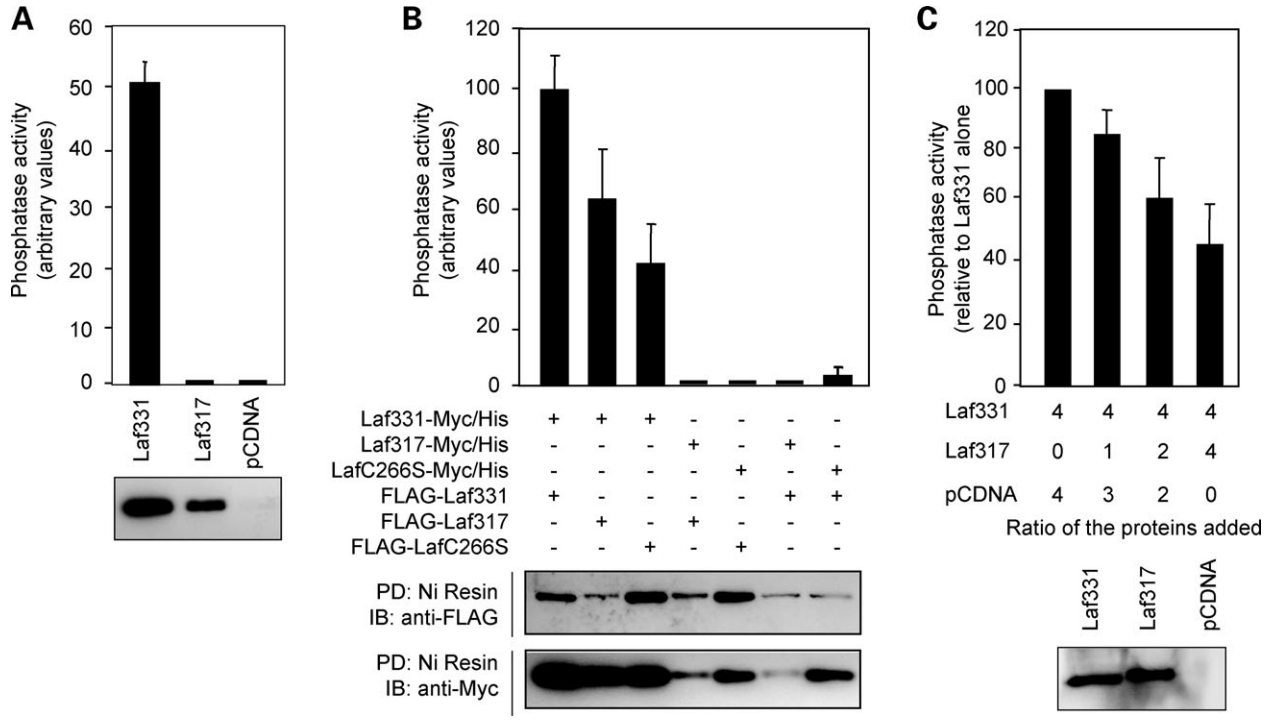

Figure 4. Laf317 is an inactive phosphatase that competes with laf331 for the substrate and regulates the activity of laf331 by forming a heterodimer. (**A**) Myc/His-tagged laf331 or laf317 was transiently expressed in COS-7 cells, affinity purified using Nickel resin and the resin-bound protein was used for the phosphatase assay using pNPP. The pull-down product from the empty vector transfected (pcDNA) cells was used as negative control. Each bar represents the mean average of three independent assays. The purity of the pulled-down protein in each experiment was examined by immunoblotting, as shown below the graph. (**B**) In order to check whether the heterodimers of laf331–laf317 are active as a phosphatase or not, COS-7 cells were transiently transfected with the expression construct as indicated and processed for the Ni-affinity resin pull-down assays. The purified proteins were used for the phosphatase assay using the pNPP as substrate and the activity was plotted on the *y*-axis. Values shown are means of three independent reactions. The purity and expression levels of the pulled-down proteins in each experiment were examined by immunoblotting, as shown below the graph. (**C**) In order to establish whether laf317 would compete with laf331 for substrate binding, Myc/His-tagged laf331 and laf317 were expressed separately in COS-7 cells, affinity purified and the two isoforms were mixed together in different ratios as indicated and assayed for phosphatase activity using pNPP. The value shown in each bar is mean of three independent reactions, and is relative to the activity shown by laf331 alone. The volume of pulled-down product added to each reaction was normalized by adding appropriate amount of resins from the lysates of cells transfected with the empty vector (pcDNA). Similarly, purity of the proteins (laf331 and laf317) used for the assay was confirmed by immunoblotting, as shown below the graph. The fractional saturation of enzyme was calculated in pilot experiments by testing various concentrations of pNPP in a reaction that had a fixed amount of enzyme. Thus, 25 mM concentration of pNPP was found to be closer to the saturation level for the amount of purified laf331 used in the phosphates assay (data not shown).

Figure 5C, when the heterodimer was pulled using a tag specific to laf331, a majority of laf331 was detected in the supernatant fraction and a smaller fraction was seen in the pellet fraction. However, laf317, which was pulled by laf331, was detected only in the supernatant fraction, suggesting that the laf331–laf317 heterodimer does not bind to glycogen. This conclusion was further strengthened when the heterodimer was pulled by laf317 and tested for its affinity to glycogen; neither laf317 nor laf331 was seen in the pellet fraction further strengthening the conclusion that the laf331–laf317 heterodimer does not bind to glycogen *in vitro* (Fig. 5C).

Malin interacts with and degrades laf317

Malin is known to co-localize, interact and degrade laf331 through the proteasomal system (4,5,23). Therefore, we were interested in testing whether or not malin would interact with and degrade laf317. For this, we co-expressed malin with laf331 or laf317 in COS-7 cells and processed for double immunofluorescence staining or for the pull down assays using FLAG-affinity resins. Laf317 showed almost complete overlap with malin in immunostaining, suggesting that laf317 might occupy the same cellular compartment as malin that allows for a physical interaction (Fig. 3K–M). Laf331 is known to form aggresome, either on its own or with malin, when cells were treated with a proteasomal blocker (5). We therefore checked whether laf317 would show the same property or not by treating the cells with MG132—a proteasomal blocker. As shown in Figure 3A–D, laf317 developed aggresome-like perinuclear aggregates upon proteasome blockade when expressed alone, and when expressed with laf331 or malin (Fig. 3H–J and N–P).

In order to check whether laf317 would physically interact with malin or not, we have co-expressed FLAG-tagged laf331 or laf317 with Myc/His tagged malin and pulled laforin isoforms using anti-FLAG antibody. As shown in Figure 6A, both isoforms of laforin were able to pull-down malin, suggesting that, similar to laf331, laf317 could serve as malin's substrate. To test this possibility, we co-expressed

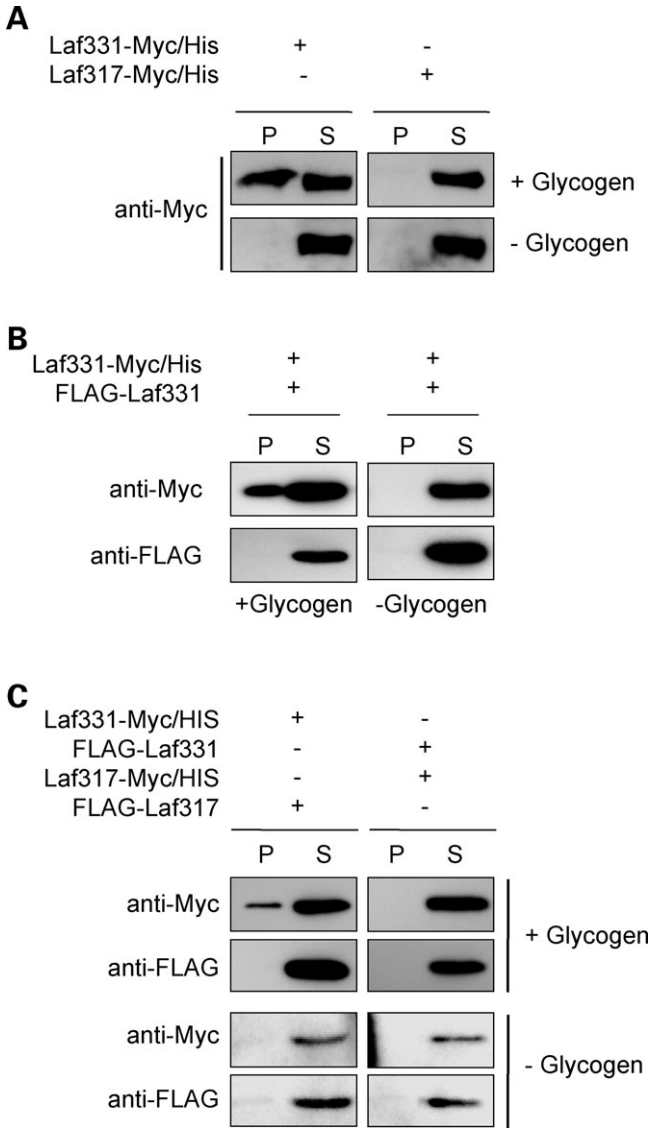

Figure 5. Laf331 binds to glycogen only as a monomeric form, whereas laf317 does not bind to glycogen as a homodimer or as a heterodimer. (**A**) Purified laf331 or laf317 protein was incubated in the presence (+) or absence (−) of glycogen, centrifuged, and the supernatant (S) and the pellet (P) fractions were collected and subjected to immunoblot analysis using appropriate antibody. (**B**) In order to establish whether laf331 binds to glycogen as a monomer or as a dimer, COS-7 cells were double transfected with plasmids that code for FLAG-tagged laf331 and Myc/His-tagged laf331, the proteins were purified using Ni-affinity resins, eluted from the resins and checked for glycogen binding affinity as mentioned above. The same blot was used for probing with anti-FLAG and anti-Myc antibodies. Control experiment lacking the glycogen was carried out in parallel. (**C**) To check whether laf317 would bind to glycogen as heterodimer with laf331, the isoforms were expressed in COS-7 cells as indicated, affinity-purified using nickel resin, eluted and checked for glycogen binding property as described above. The proteins in the pellet (P) and supernatant (S) fractions were processed for immunoblotting with anti-FLAG and anti-Myc antibodies. Control experiment lacking the glycogen was carried out in parallel.

laf317 with wild-type malin, or with a catalytically inactive mutant malin (C26S) (4,5) in COS-7 cells and evaluated the relative levels of laf317. As a control experiment, in parallel, cells expressing laf317 and wild-type malin were treated with a proteasomal blocker (MG132) (Fig. 6B). Identical set of experiments were also carried out with laf331, and the total cell lysates were immunoblotted to detect the relative levels of laf317 or laf331 (Fig. 6B). Laf317 showed significant reduction in their cellular levels when expressed with the wild-type malin when compared with those that were expressed with the malin mutant, C26S (Fig. 6B). Significant increase in the levels of misfolded proteins was observed when cells co-expressing wild-type malin were treated with a proteasomal inhibitor (MG132), suggesting that malin facilitates the degradation of laf317 through the proteasomal system (Fig. 6B). Identical results were also obtained for laf331, suggesting that both the isoforms of laforin serve as substrates for malin (Fig. 6B). To confirm whether malin show equal preference to the laforin isoforms, or whether it prefers one over the other, we expressed the catalytically inactive malin mutant C26S with laf331 and laf317, and pulled malin and immunoblotted for laforin isoforms (Fig. 6C). While the intensity of the signals for laf331 and laf317 was almost same in the whole cell lysate, the intensity of signal for laf331 in the pulled down product was almost two-fold higher than that of laf317, suggesting that malin could display higher affinity towards laf331 (Fig. 6C). Since the pull-down assays were done by using a catalytically inactive malin, and were repeated twice, we assume that the intensity of the signal corresponds to the total protein available for interaction and does not represent the amount of product that 'escaped' malin-mediated degradation.

Role of laf317 in LD pathology: effect of laf331-specific mutations on laf317

Among the two known isoforms of laforin, laf331 is conserved across the vertebrates (11). Splice variants of laforin are not seen in mice or other mammalian species, and thus, laf317 appears to be unique to human (17, Dubey and Ganesh, unpublished observations). Since laf317-specific mutations have not yet been identified in LD patients, and because knockout of laf331 led to the LD-like phenotype in mice (24), it has been suggested that laf317 may not be relevant to LD pathology in humans (1,17,18). With the demonstration of formation of functional heterodimers between laf331 and laf317 in the present study, we were tempted to check the possibility of impairment in the interaction between a mutant laf331 and the wild-type laf317. For this, we selected two LD-associated mutations, Q319fs and L310W, and these two mutations are intronic in the transcript that encodes laf317 and, therefore, unlikely to alter the peptide sequence of laf317 (17,18) (Fig. 7A). We created these mutations in the laf331 coding sequence and expressed them in-frame to a Myc/His-tag at the carboxyl terminal. We have examined whether the two mutants would form homodimers by chemical cross linking with DSS and evaluated the cross-linked products by immunoblot analysis. As shown in Figure 7B, both the mutants have formed a higher molecular weight band in the cell lysates that were treated with the cross-linker, suggesting that the mutant laf331 could form homodimers with itself. We next checked the possibility whether or not these mutants interact with laf317 or the

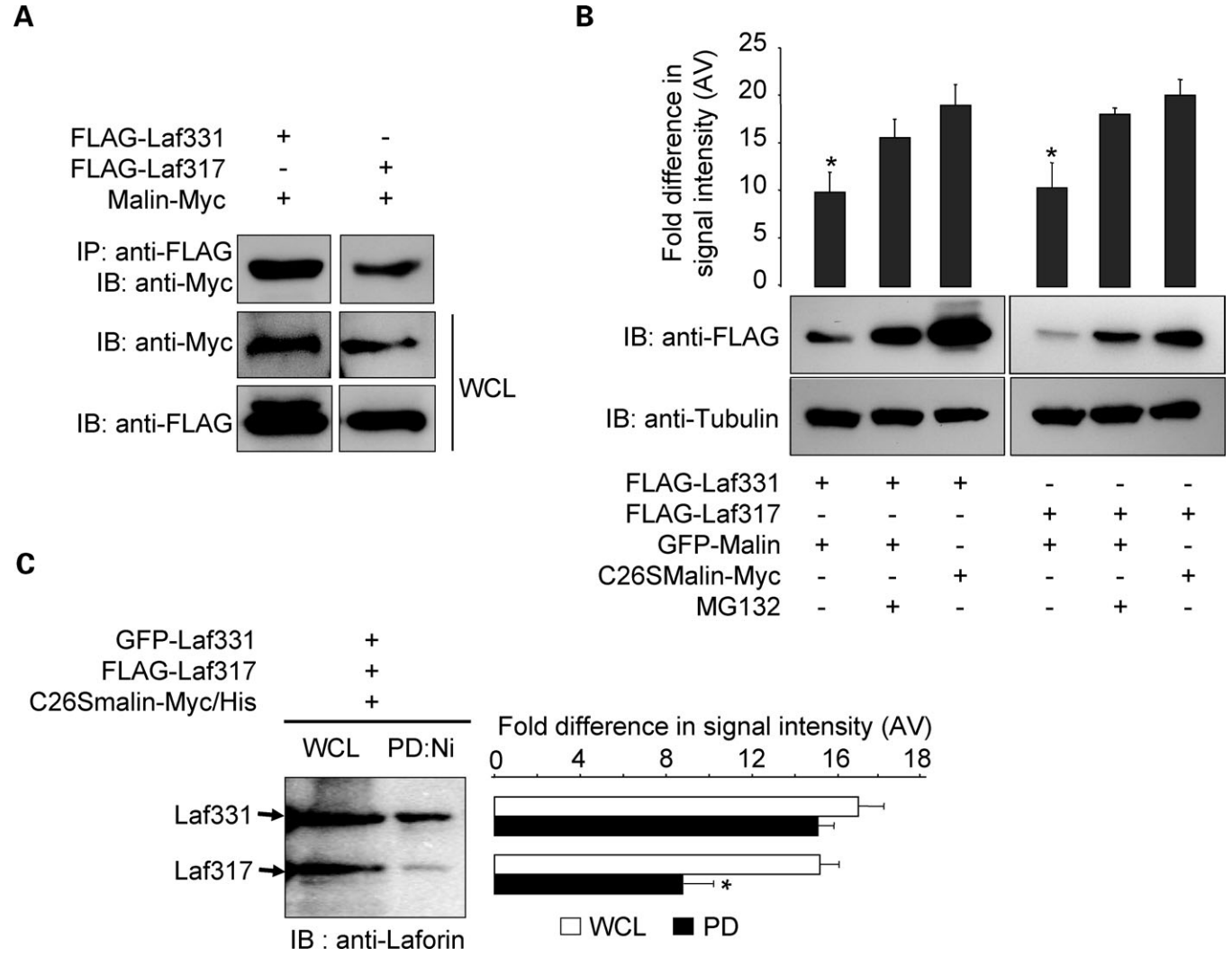

Figure 6. Malin interacts with and degrades laf317. (**A**) To check the interaction of malin with laf317, COS-7 cells were co-transfected with constructs that code for Myc-tagged malin and FLAG-tagged laf317, and processed for immunoprecipitation assays using anti-FLAG resin. Cells that were transfected with laf331-Myc and FLAG-malin were processed in parallel as positive controls. The pulled-down products (IP) and whole cell lysates (WCL) were immunoblotted (IB) with anti-FLAG and anti-Myc antibodies. (**B**) To check whether malin promotes degradation of laf317, COS-7 cells were co-transfected with expression constructs for wild-type or mutant malin and laforin isoforms as indicated, and the cellular levels of laf317 or laf331 was established by immunoblotting. As a control, cells that expressed wild-type malin and laf331 or laf317 were treated with MG132 and processed in parallel. The signal intensity of laforin isoforms was measured and plotted as a bar diagram. Values shown are mean of two independent experiments, and an asterisk mark indicates the significant decrease in the signal intensity when compared with the other two lanes (mutant malin or the MG132 treatment) as calculated by paired *t*-test. Immunoblotting for γ-tubulin levels served as loading control. (**C**) To check relative affinity of laf331 and laf317 towards malin, COS-7 cells were transiently transfected with constructs that express Myc/His-tagged mutant malin (C26S) with GFP-laf331 and FLAG-laf317 and processed for Ni-affinity pull-down assays. The whole cell lysate (WCL) and the pulled-down (PD) samples were run in the same gel and processed for immunoblotting with anti-laforin antibody that detects both isoforms of laforin (identified by arrows on the left). The relative intensity of signals detected for the whole cell lysate and the pulled-down product was estimated and plotted. The value in the bar represents mean of two independent experiments, and an asterisk mark indicates the significant decrease in the signal intensity when compared with the whole cell lysate (WCL). That the GFP-tag did not alter the affinity of laf331 towards malin was established by pull-down assays (pilot experiments) in which malin was unable to pull GFP when co-expressed and malin was able to pull FLAG-tagged laf331 and GFP-tagged laf331 with equal affinity when all three were expressed together (data not shown). Numbers given on the *y*-axis in both (B) and (C) represent arbitrary values (AV).

wild-type laf331. For this, the mutants were co-expressed with FLAG-tagged laf331 or laf317, pulled-down using Ni-resin and detected using a tag-specific antibody. As can be seen in Figure 7C, laf331, but not laf317, was pulled-down by Q319fs and L310W mutants, suggesting that these mutations have abolished the interaction between laf331 and laf317. Thus, in these patients, the formation of laf331–laf317 heterodimers is unlikely to take place.

DISCUSSION

Alternative splicing in the human *EPM2A* gene gives rise to two mRNA variants. Here we demonstrate that the two isoforms of laforin, coded by the transcript variants, are functionally distinct from each other. Laf317 is an inactive phosphatase and acts as a dominant-negative regulator for the phosphatase activity of laf331. We further show that

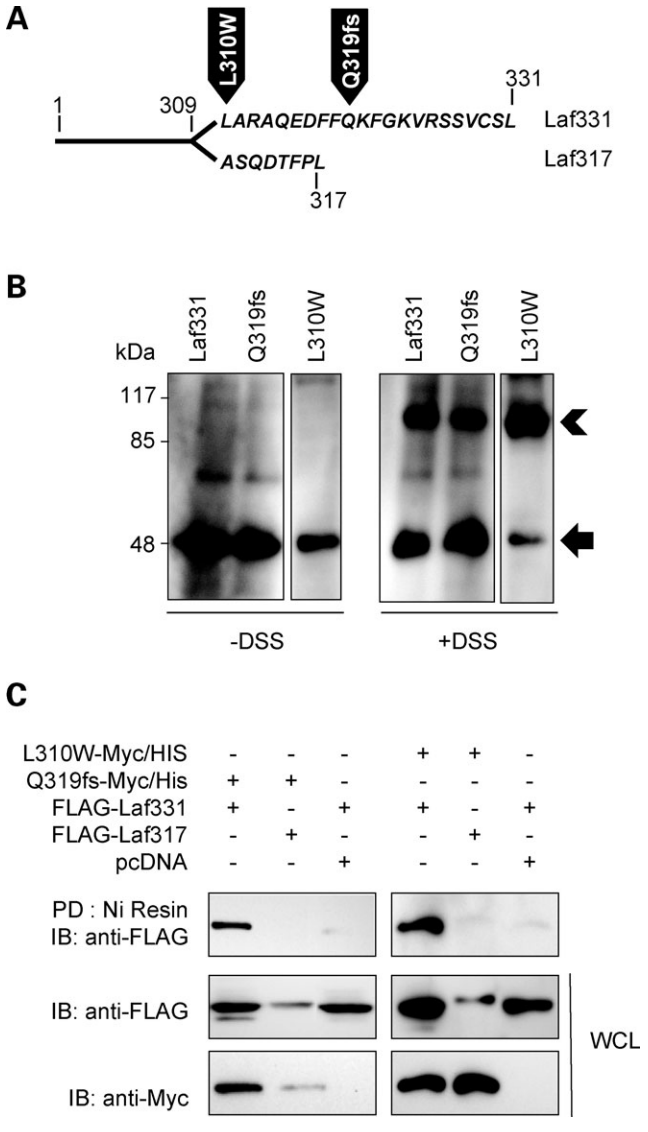

Figure 7. LD-associated mutations affect interaction between laf331 and laf317. (**A**) Schematic diagram showing the carboxyl-terminal unique sequence of laf331 and laf317, and the positions of the two mutations (L310W and Q319fs) whose effects are restricted to laf331. (**B**) The wild-type form and the two mutant forms of laf331 were transiently expressed in COS-7 cells, treated (+) or not treated (−) with DSS and subjected to immunoblotting with anti-laforin antibody. Similar to the wild-type form, both the mutants formed homodimers upon DSS treatment (identified by an arrowhead). The monomeric forms are identified by an arrow. (**C**) To check the physical interaction between laf331 mutants and laf317, COS-7 cells were transiently transfected with expression constructs as indicated and processed for pull-down assays using Ni-affinity resin. Interactions between proteins were tested by probing the pull-down products (PD) and the whole cell lysates (WCL) with appropriate antibodies. Cells that were transfected with a construct that codes for the FLAG-tagged protein and an empty vector served as negative controls.

each isoform of laforin can form a homodimer with itself or a heterodimer with the other. The pull-down and cross-linking assays used in the present investigation for demonstrating the dimerization potential of laf317 and laf331 are indirect approaches, and therefore one cannot exclude the possibility that the interaction with itself or with the other isoform requires additional protein(s) to form the complex. Nonetheless, we believe, for the following reasons, that the laforin isoforms might directly interact with each other and form heterodimers; (i) the 'dimeric band' detected in the immunoblot for the cross-linked samples for both isoforms are of the expected size for the dimers, (ii) a recent study has demonstrated that the bacterially expressed laf331 dimerize and is enzymatically active (20), and (iii) laf331 and laf317 interact with each other in a yeast two-hybrid screen (our unpublished observations). Similar to laforin, functional isoforms of protein phosphatases that result from differential splicing are known in the literature. For example, differential dimerization properties of splice variants negatively regulate the activity of CD45, PTPε and PPARγ1 phosphatases and their signaling cascades (25–28).

We cannot yet ascribe any physiological significance to the homo- and the heterodimerization properties of laf331 and laf317 because the phosphatase assays employed in the present study were limited to a synthetic substrate. Since dimerization is required for the phosphatase activity of laf331, we nonetheless believe that some as yet unidentified additional stimuli may affect dimerization property and modulate the activity of laf331 to the desired level. One of them could be the glycogen level. Since the dimeric form of laf331 does not bind to glycogen *in vitro*, we speculate that the activity of laf331 could be modulated by the relative levels of free versus glycogen-bound forms of laf331. Thus, the monomeric laf331 that is bound to the glycogen could be an inactive species; conversely, the affinity towards glycogen could be lower for the enzymatically active dimeric form of laf331. A support for this notion comes from the report of Wang *et al*. (20), wherein, they have shown that the phosphatase activity of laf331 was profoundly inhibited by the addition of polysaccharides. This binding, and hence the dimerization process, is likely to be dynamic in nature because laf331 is also known to remove the phosphate groups in complex polysaccharides (22,29), and loss of laforin lead to increased phosphorylation of glycogen in laforin deficient mice (29). However, it is unclear as of now as to whether the dimeric laf331 would dephosphorylate the phospho groups before binding to the polysaccharide moieties as a monomeric form, or whether its affinity towards polysaccharides as monomeric form would facilitate its physical interaction leading to dimerization and eventual dephosphorylation of polysaccharides. Clearly, the dimerization property of laf331, and laf317, in relation to glycogen affinity needs to be studied further under *in vivo* conditions.

Our observations that laf317 does not bind to glycogen are intriguing because the carbohydrate binding domain is located in the amino-terminal of the peptide and its sequences are not altered by the differential splicing of the transcript that encodes laf317. It is likely, therefore, that the unique carboxyl-terminal domain affects the conformation of laf317 such that laf317 is unable to bind to the glycogen. Indeed missense mutations in the phosphatase domain are known to affect the glycogen affinity of laf331 (10), suggesting that the laforin-glycogen interaction is conformation-dependent. Clearly, structural studies would provide insights into the conformational changes of the isoforms resulting from the alternative splicing.

Although we have not yet checked the temporal expression pattern of the two isoforms of laforin in various human tissues, we have shown here that if present together, laf317 affects the phosphatase activity of laf331 in a dominant-negative manner, at least with a synthetic substrate tested. This property of laf317 is analogous to the one described for a splice variant of PPARγ1 in activating a signaling cascade (27). Protein isoforms that are encoded by alternative splice variants that exert repressive activity over other species are known for many proteins, especially in the class of nuclear receptors (30–32). The protein species of splice variants with distinctive, overlapping and in some instances opposite functions to the major isoforms of a gene product may serve as a general mechanism by which the cellular functions of a protein complex can be modulated in response to various physiological signals. For example, the laforin-mediated cellular pathway could be altered by variations in the ratio of laf331–laf317 isoforms because the dephosphorylation of a laforin substrate might depend upon the cellular levels of laf331 homodimers. Thus, an increase in the levels of laf317 variant could sequester laf331 into the heterodimer, making it an enzymatically inactive complex. It has been shown previously that the expression levels of laf317 transcript in human brain tissues was a few fold lower when compared with that of laf331 (16). It is likely that the physiological signals that modulate the cellular levels of laforin isoforms might act either at the level of transcript (splicing) or protein (synthesis and/or degradation). We show here that one of such factors could be the malin ubiquitin ligase, because malin interacts with and degrades both laf331 and laf317. In this regard, it is intriguing to note that, despite almost complete co-localization with laf317 (nuclear and cytoplasmic localization), malin displays higher affinity towards laf331 though the latter is restricted to the cytoplasm in localization. The unique carboxyl-terminal of laf317 might alter its interaction/affinity with malin because missense mutations in laf331 are known to impair the interaction between laf331 and malin (4,14). It is likely, therefore, that the malin-mediated protein turn-over rates could be different for the monomers of laf331 and laf317, their dimers and for the laf331–laf317 heterodimers. The relative ratios of these different forms in cellular milieu could also be altered by cellular signals, and thus, malin could prefer one complex over the other for the degradation. Thus malin, by selectively degrading a given isoform or its complex, might either unmask or promote the inhibitory effect of laf317 over laf331. On the other hand, laforin isoforms could also alter the cellular functions of malin. We have shown recently that laf331 co-localize with malin in the aggresome structures, and proposed that the laf331–malin complex might enhance the ubiquitination of its substrates and facilitate their efficient degradation (5). We show here that both laf331 and laf317 target aggresome upon proteasome blockade, and that both isoforms co-localize with malin. Thus, as it has been demonstrated here for the phosphatase activity and glycogen affinity, laf317 could compete with laf331 and block the degradation of a target protein by the malin–laf331 complex. For example, malin is known to degrade PTG in a laf331-dependent manner and thus loss of laf331 leads to excessive glycogen deposition (13,14). It would, therefore, be of interest to check whether laf317 competes with laf331 for the interaction with PTG and prevents its degradation or not. Intriguingly, it was shown that a catalytically inactive mutant of laf331 can promote the degradation of PTG (13,14). Therefore it is likely that laf317 would do the same function if it interacts with PTG, because the phosphatase activity is not likely to be required for this process. It should be noted, however, that a majority of these experiments was carried out in non-human cell lines which do not have laf317 isoform and hence the relevance of these findings should be tested in human cell lines to understand the role of laf317 in PTG stability. A second possibility could be that laf317 competes with, and blocks laf331 from dephosphorylating the glycogen complex, because laf331 is known to remove phosphate groups from glycogen (22,29), and the phospho content of glycogen has gone up several fold in the laforin deficient mice (29).

A large number of mutations have been identified in the *EPM2A* gene that are known to or expected to affect the functional properties of laforin protein (reviewed in 1). Among these, the two mutations, Q319fs and L310W, characterized in the present study offer novel insights into the LD pathogenesis (18). Since the effects of these mutations are restricted to the laf331 isoform, it was suggested that the isoform laf317 might not have any relevance to LD pathogenesis (17,18). We show here that both the mutations affected the interaction between mutant laf331 and laf317, thereby suggesting that, although the mutation is specific to laf331, the functional properties of the laf331–laf317 heterodimers might have been affected in these patients. Thus, defects in one isoform are likely to affect at least some of the functions of the other isoform. In this regard, it is interesting to note that unlike laf331, laf317 is localized in the nucleus as well. Thus, laf317 might have some unique functions that are not likely to be compensated by laf331. Since mutations that exclusively affect laf317 are yet to be identified, the physiological impact of loss of laf317 in neurological functions is difficult to speculate. It would be of interest, therefore, to develop appropriate models to explore the shared and distinct functions of laforin isoforms and their regulatory roles on malin-mediated cellular cascade. Ongoing studies in our laboratory are expected to shed light on some of these issues.

MATERIALS AND METHODS

Expression constructs

The expression vectors containing Myc- or GFP-tagged wild-type or mutant forms of laforin or malin were described previously (5,18,19). For FLAG-tagged constructs, the coding region of laf331 was cloned in-frame with a FLAG epitope in a pcDNA vector. For laf317 expression constructs, the image clone 266552, obtained from Open Biosystem, was used as a template for PCR amplification and cloned into expression constructs as described previously (16). The laforin mutant clone, Q319fs, was generated by creating the mutation in coding regions of laf331 by site-directed mutagenesis as described earlier (7). The mutant clone L310W was described in one of our previous studies (18).

Cell culture and transfections

COS-7 cells were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich India Pvt Ltd) supplemented with 10% (vol/vol) fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were grown at 37°C in 5% CO_2. Transfections were performed using LipofectAMINE 2000 (Invitrogen Inc, USA) or Escort V (Sigma-Aldrich India Pvt Ltd) transfection reagent according to the manufacturer's protocol. Cells were harvested at 24 h (LipofectAMINE 2000) or 36 h (Escort V) post-transfection, as recommended by the manufacturer.

Immunostaining and antibodies

COS-7 cells, grown on gelatin-coated sterile glass coverslips, were fixed and processed for immunofluorescence microscopy essentially as described earlier (5). Cells were fixed with paraformaldehyde (4%), permeabilized (0.05% Tween 20) and subsequently incubated with primary and secondary antibodies. For nuclear staining, fixed cells were incubated with 10 μM 4′, 6-diamidino-2-phenylindole (DAPI). Fluorescence images were obtained using a fluorescence microscope (Nikon Eclipse 80i, Japan) with 40× objective lens and were processed using ImageExpress software (Media Cybernetics, USA). Representative images were then imported to Photoshop (Adobe) for processing. The following antibodies were used in the present study; anti-Myc (Cell Signaling Technology, USA), anti-γ-tubulin, anti-FLAG (both from Sigma-Aldrich India Pvt Ltd) and anti-laforin antibody (5). Secondary antibodies were obtained from Jackson Immuno Research Inc. (USA).

DSS cross-linking

COS-7 cells that had expressed desired proteins were treated with 5 mM DSS (Pierce, Rockford, USA) for 2 h at 4°C, and then the activity was quenched with 25 mM Tris-Cl (pH 7.5), as recommended by the manufacturers. The cells were then washed briefly in ice-cold PBS, lysed in Laemmli buffer and the lysates were processed for immunoblotting.

Immunoblotting analysis

Protein samples were run on a 10% SDS–PAGE and transferred onto a nitrocellulose filter (MDI, India) as described previously (5). After blocking with 5% non-fat dry milk powder, the membranes were processed through sequential incubations with primary antibody followed by secondary antibody. Immunoreactive proteins on the filter were visualized using a chemiluminscent detection kit (SuperSignal West PICO, Pierce, USA). For quantitation, the signal intensity in the digital images was measured using the Quantity One Discovery Series software of Bio-Rad Laboratories.

Pull-down assays

To establish the physical interaction between laforin isoforms and other target proteins, we have used two different approaches. In one, the lysates of cells that had over-expressed poly-histidine-tagged protein was incubated with Ni-affinity resin (Sigma-Aldrich India Pvt Ltd) for 2 h at 4°C and processed for pull-down assays as recommended by the manufacturer. In the other method, FLAG-tagged proteins were selectively captured using the anti-FLAG M2 affinity gels (Sigma-Aldrich India Pvt Ltd) as recommended by the manufacturer. Pulled-down products were detected by immunoblotting using specific antibodies.

Phosphatase activity assay

Histidine-tagged laf331, its mutants, laf317, or their combinations as specified were transiently expressed in COS-7 cells and purified using nickel affinity resin (Sigma-Aldrich India Pvt Ltd) as per the manufacturer's protocol. Around 100 ng of the bead-bound proteins were used for each of the phosphatase assay. The reaction was performed in 75 μl reaction buffer (50 mM HEPES pH 6.0, 50 mM NaCl, 5 mM EDTA and 50 mM β-mercaptoethanol) containing 25 mM of pNPP and the reaction was carried out at 37°C for 30 min. The reaction was arrested with the addition of NaOH and absorbance was taken at 405 nm. A fraction of the bead-bound protein was boiled in Laemmli buffer and used for immunoblot analysis. The fractional saturation of enzyme was calculated in pilot experiments by testing various concentrations of pNPP in a reaction that had a fixed amount of enzyme. Thus, 25 mM concentration of pNPP was found to be closer to the saturation level for the amount of laf331 protein used in the assay (data not shown).

Glycogen binding assay

Histidine-tagged laf331 or laf317, or their combinations as specified, were transiently expressed in COS-7 cells and purified using nickel affinity resin (Sigma-Aldrich India Pvt Ltd) as per the manufacturer's protocol. The bead-bound proteins were eluted in glycogen binding assay buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% β-mercaptoethanol and 0.1 mg/ml BSA) in the presence of 20 mM imidazole. The eluted proteins were then dialyzed overnight in the glycogen binding buffer lacking imidazole and processed for glycogen binding assay as described previously (11). For each assay, 10 mg/ml glycogen was used and the protein was incubated with glycogen at 4°C for 30 min and then centrifuged at 17 000 *g* for 90 min at 4°C to get supernatant and pellet fractions. Protein samples derived from these two fractions were subjected to immunoblotting to assess the affinity of laforin isoforms towards glycogen.

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