Regulation of glycogen synthesis by the laforin–malin complex is modulated by the AMP-activated protein kinase pathway

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Lafora progressive myoclonus epilepsy (LD) is a fatal autosomal recessive neurodegenerative disorder characterized by the presence of glycogen-like intracellular inclusions called Lafora bodies. LD is caused by mutations in two genes, EPM2A and EPM2B, encoding respectively laforin, a dual-specificity protein phosphatase, and malin, an E3 ubiquitin ligase. Previously, we and others have suggested that the interactions between laforin and PTG (a regulatory subunit of type 1 protein phosphatase) and between laforin and malin are critical in the pathogenesis of LD. Here, we show that the laforin–malin complex downregulates PTG-induced glycogen synthesis in FTO2B hepatoma cells through a mechanism involving ubiquitination and degradation of PTG. Furthermore, we demonstrate that the interaction between laforin and malin is a regulated process that is modulated by the AMP-activated protein kinase (AMPK). These findings provide further insights into the critical role of the laforin–malin complex in the control of glycogen metabolism and unravel a novel link between the energy sensor AMPK and glycogen metabolism. These data advance our understanding of the functional role of laforin and malin, which hopefully will facilitate the development of appropriate LD therapies.

INTRODUCTION

Lafora progressive myoclonus epilepsy (LD, OMIM 254780) is an autosomal recessive neurodegenerative disorder characterized by the presence of glycogen-like intracellular inclusions named Lafora bodies (1–5). LD is a fatal disorder that occurs worldwide, but is relatively more frequent in Mediterranean countries. LD initially manifests during adolescence with generalized tonic-clonic seizures, myoclonus, absences, drop attacks or visual hallucinations. As the disease proceeds, a rapidly progressive dementia with apraxia, aphasia and visual loss ensues, leading patients to a vegetative state and death, usually within the first decade from onset of the first symptoms (6,7). Mutations have been identified in two genes, EPM2A

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that laforin interacted physically with malin, in agreement with recent reports (17,28). Moreover, the laforin binding site was located within the four C-terminal NHL domains of malin (residues 208–395) (data not shown). Similarly, functional analysis of several laforin and malin mutations identified in patients with LD indicated that the formation of the laforin–malin complex and its interaction with PTG is crucial in LD pathogenesis (Supplementary Material, Fig. S1). Since laforin forms stable complexes with malin and also interacts with PTG, we tested whether malin also interacted with PTG. Although, we could not detect a direct interaction between malin and PTG, a robust two-hybrid interaction between PTG and malin was observed when laforin was overexpressed in these assays (Fig. 1). These results suggest the formation of a possible ternary complex in which laforin would tether the interaction between malin and PTG. Similar results were obtained with laforin C266S, an artificial laforin mutant that interacts properly with malin, but lacks phosphatase activity (Supplementary Material, Fig. S1), indicating that the formation of ternary complexes between laforin, malin and PTG did not require the phosphatase activity of laforin (Fig. 1).

The laforin–malin complex prevents glycogen accumulation caused by overexpression of PTG in FTO2B hepatoma cells, ubiquitinates PTG and targets it for degradation

Recently, we described a novel mechanism for the regulation of glycogen synthesis that involves the laforin–malin complex in neurons. This regulatory mechanism controls the levels of GS and PTG via a proteasomal degradation pathway (30). Because disturbance of this novel glycogen regulatory mechanism, as a consequence of mutations in laforin or malin, may explain the generation of the glycogen-like intracellular inclusions (Lafora bodies) present in all tissues of LD patients, we determined whether this glycogen regulatory mechanism also operates in tissues that normally synthesize glycogen, such as liver. Using FTO2B hepatoma cells as a model system, we observed that treatment of these cells with increasing amounts of adenovirus expressing a GFP–PTG fusion protein (Ad–GFP–PTG) resulted in a dose-dependent enhancement of glycogen accumulation (Fig. 2A). The PTG-induced glycogen accumulation was, however, progressively prevented by the co-infection of FTO2B cells with increasing amounts of adenovirus expressing laforin (Ad–laforin) and malin (Ad–malin) (Fig. 2B). This effect was dependent on the presence of both laforin and malin, since in the absence of one of them, no inhibition of the glycogenic properties of PTG was observed (Fig. 2B). The co-expression of laforin and malin with GFP–PTG in FTO2B cells resulted in a drastic reduction in the levels of GFP–PTG (Fig. 2C). The levels of laforin were also diminished, in agreement with a recent report indicating that malin interacts with and ubiquitinates laforin, leading to its degradation (28). However, the co-expression of laforin and malin did not change the total levels of GS in FTO2B cells. This result is consistent with the observation that only the muscular GS isoform (MGS), but not the liver isoform (LGS), is degraded by the overexpression of laforin and malin in neuron cells (30) and unpublished data, suggesting the existence of tissue-specific differences in the regulation of glycogen synthesis by the laforin–malin complex. We also found that the levels of accumulated glycogen
correlated directly with the activity of GS. We observed an increase in the GS activity ratio (Glu-6P/Glu-6P) when the cells were infected with Ad–GFP–PTG adenovirus (ratio of 0.46 versus 0.13, observed in cells infected with Ad–GFP adenovirus) and a decrease in the GS activity ratio when the cells were co-infected with Ad–GFP–PTG, Ad–laforin and Ad–malin (ratio of 0.34). Consistent with these results, we observed a partial recovery of the phosphorylated status of GS at Ser461 when the cells were co-infected with Ad–GFP–PTG, Ad–laforin and Ad–malin adenovirus (a sign of GS inactivation). These results indicated an inhibition of the dephosphorylating activity of PTG under the later conditions, possibly as a consequence of lower levels of this protein (Fig. 2C).

To determine whether the reduction in the levels of PTG was due to an increased ubiquitin-dependent proteasomal degradation, mediated by the laforin–malin complex, we expressed in HEK293 cells a myc-tagged form of PTG (myc-PTG) and a modified form of ubiquitin (tagged with 6xHis residues), which allowed the purification of ubiquitin-tagged proteins by metal affinity chromatography (TALON column; see Materials and Methods). As observed in Figure 3, in the bound fraction of the TALON column, the anti-myc antibody detected a polydispersed high molecular weight material (lanes 2 and 3), which was absent in the cells that expressed myc-PTG, but not the modified form of ubiquitin (lane 1), indicating that myc-PTG was ubiquitinated in vivo (Ub-myc-PTG). Interestingly, the co-expression of laforin and malin improved the ubiquitination of myc-PTG (lane 3) in comparison with cells that only co-expressed malin (lane 2) or with cells that did not co-express laforin and malin (data not shown). No high molecular weight forms of myc-PTG were observed in the crude extracts, possibly due to the low abundance of these forms due to their rapid degradation. These results suggest that the laforin–malin complex modified PTG and targeted it for ubiquitin-dependent proteasomal degradation. The affinity column also retained unspecifically the unmodified form of myc-PTG, perhaps due to the polysaccharide binding domain.

Figure 1. Laforin tethers the interaction between PTG and malin. Yeast CTY10-5d strain was transformed with plasmids pEG202-PTG (LexA.PTG), pACT2-malin (GAd–malin) or pACT2 (GAD, empty plasmid) and plasmids pSK93 (empty), pSK-laforin or pSK-laforin C266S. Transformants were grown until exponential phase (A600 0.5) in selective SC medium containing 4% glucose. Protein interaction was estimated using the yeast two-hybrid system, by measuring the β-galactosidase activity. Values correspond to means from 4 to 6 different transformants (bars indicate standard deviation), **P < 0.01.

Figure 2. Laforin–malin complex counteracts the glycogenic effect of PTG. (A) Rat hepatoma FTO2B cells were infected with increasing amounts of Ad–GFP–PTG adenovirus or with 30 μl of Ad–GFP adenovirus. Twenty-four hours after the infection, the amount of glycogen was determined as described in Materials and Methods. Bars indicate standard deviation of three independent experiments (**P < 0.01; ***P < 0.001). (B) FTO2B cells were infected with 300 μl of Ad–GFP–PTG and increasing amounts of Ad–laforin and Ad–malin adenovirus. Twenty-four hours after the infection, the amount of glycogen was determined as described in Materials and Methods. Bars indicate standard deviation of at least three independent experiments (***P < 0.001). (C) Cell extracts (60 μg) from FTO2B cells treated as in section (B) were obtained and analyzed by western blotting using anti-GFP, anti-GS, anti-phospho Ser461 GS, anti-actin, anti-laforin or anti-HA antibodies (Ad–malin adenovirus produces an N-terminal HA-tagged malin).
The Snf1 kinase (between laforin and malin, we used a yeast mutant lacking comparable to those observed in low (0.05%) glucose (Fig. 4C). An activated form of the catalytic subunit of mammalian AMPK increased the interaction between laforin and malin in high (4%) glucose conditions, to levels [KD T172D; (40,41)] increased the interaction between laforin and malin in the presence of low (0.05%) glucose conditions (Fig. 4B). Concurrently, transformation of wild-type yeast cells with a constitutively activated form of the catalytic subunit of mammalian AMPK [KD T172D; (40,41)] increased the interaction between laforin and malin in high (4%) glucose conditions, to levels comparable to those observed in low (0.05%) glucose (Fig. 4C).

In contrast to other eukaryotes, yeast accumulates glycogen when growing under low glucose conditions. To demonstrate that the increase in the interaction between laforin and malin was due to the action of Snf1/AMPK proteins and not to the accumulation of glycogen, we measured the interaction in a gac1Δ yeast mutant, lacking the main regulatory subunit of the PP1 (yeast PTG orthologue), that targets the phosphatase catalytic subunit to substrates involved in glycogen metabolism. This mutant is unable to accumulate glycogen, whereas the Snf1 pathway is still activated upon growth in low glucose conditions (42). As shown in Figure 4B, the interaction between laforin and malin increased in the gac1Δ mutant when growing in low (0.05%) glucose conditions, as observed in the wild-type control. In addition, we did not observe any accumulation of glycogen in cells growing in high (4%) glucose and expressing the plasmid pSK-KDT172D (data not shown). These data suggested that the increase in the interaction between laforin and malin was related to the action of Snf1/AMPK proteins and not to the accumulation of glycogen in yeast cells. This modulation seems specific for the interaction between laforin and malin since AMPK activation did not affect the interaction between laforin and PTG (data not shown).

AMPK interacts and phosphorylates laforin in vitro

AMPK is a heterotrimer comprised of a catalytic subunit (α), a scaffolding subunit (β) also involved in substrate recognition, and a regulatory subunit (γ). To characterize the potential interaction between laforin and/or malin and AMPK, we performed a yeast two-hybrid analysis with the α2, β2 and γ1 AMPK subunits, the most abundant isoforms found in liver and skeletal muscle. These analyses showed that laforin interacted with the catalytic AMPKα2 and the AMPKβ2 scaffolding subunits, but not with AMPKγ1 (Fig. 5A). These interactions were not regulated by the level of glucose (data not shown). In contrast to laforin, malin did not interact with any of the AMPK subunits (Fig. 5A) (an empty LexA plasmid in combination with the three AMPK subunits gave less than 1 Unit of β-galactosidase activity; data not shown). We confirmed that laforin interacted with AMPK in vivo by co-immunoprecipitation experiments using HEK293 cells transfected with pCINeo::laforin and pCMV-HA-AMPKα2. As shown in Figure 5B, cell extracts immunoprecipitated using an anti-HA monoclonal antibody (to immunoprecipitate AMPKα2) co-immunoprecipitated laforin. We next analyzed whether purified AMPK could phosphorylate GST::laforin in vitro. These in vitro experiments, in addition to further corroborating the interaction between laforin and AMPK, demonstrated that AMPK was able to phosphorylate recombinant GST::laforin produced in bacteria (Fig. 5C). Taken together, these data suggest that AMPK might be involved in the regulation of the laforin–malin complex.

A dominant negative form of AMPK impairs the effect of the laforin–malin complex on the glycogenic activity of PTG

Following our observations that the laforin–malin complex downregulates the glycogenic activity of PTG and that the

Figure 3. PTG is ubiquitinated in vivo. HEK293 cells were transfected with pCMV-myc-PTG and with the indicated combinations of pCMV-UBiquitinHis, pcDNA3-HA-malin and pCINeo::laforin plasmids. Thirty-six hours after transfection, cells were broken in lysis buffer containing guanidine–HCl (see Materials and Methods) and 4 mg of protein of clarified extracts were loaded on a TALON column. The column was extensively washed and finally eluted with 2x Laemmli sample buffer. Hundred microgram of clarified extracts and the eluted fraction from the TALON column were analyzed by immunoblotting using anti-myc antibodies. The unmodified myc-PTG protein is retained unspecifically in the TALON column (asterisk). The laforin–malin interaction is modulated by the AMP-activated protein kinase

Since laforin and malin formed a functional complex (discussed earlier), we studied next how the formation of this complex could be regulated. Two-hybrid analysis of yeast cells co-transformed with laforin and malin demonstrated a three-fold increase of the laforin–malin interaction when the cells were incubated in low (0.05%) glucose containing medium (Fig. 4A). These conditions in yeast determine activation of the Snf1 kinase, the orthologue of the human catalytic subunit of the AMPK, a metabolic-sensing protein that plays a key role in maintaining the cellular energy balance (31). AMPK is a serine/threonine protein kinase that acts as a sensor of the cellular energy status. Once activated, it switches on catabolic pathways and switches off many ATP-consuming processes including anabolic pathways [see (32–35), for review]. Activation of AMPK requires phosphorylation of the α catalytic subunit by an upstream kinase, with LKB1 and CaMKKβ sharing this role (36–39).

To test whether the Snf1 kinase modulated the interaction between laforin and malin, we used a yeast mutant lacking the Snf1 kinase (snf1Δ) and observed that the interaction between laforin and malin did not increase in the presence of low (0.05%) glucose conditions (Fig. 4B). This mutant is unable to accumulate glycogen, whereas the Snf1 pathway is still activated upon growth in low glucose conditions (42). As shown in Figure 4B, the interaction between laforin and malin increased in the snf1Δ mutant when growing in low (0.05%) glucose conditions, as observed in the wild-type control. In addition, we did not observe any accumulation of glycogen in cells growing in high (4%) glucose and expressing the plasmid pSK-KDT172D (data not shown). These data suggested that the increase in the interaction between laforin and malin was related to the action of Snf1/AMPK proteins and not to the accumulation of glycogen in yeast cells. This modulation seems specific for the interaction between laforin and malin since AMPK activation did not affect the interaction between laforin and PTG (data not shown).
AMPK regulates the interaction between laforin and malin. (A) The laforin–malin interaction is enhanced by low glucose. Yeast CTY10-5d strain was transformed with plasmids pEG202-laforin (LexA-laforin) and pACT2-malin (GAD–malin) or the empty plasmid pACT2 (GAD). Transformants were grown until exponential phase (A600 0.5) in selective SC medium containing 4% glucose, and then washed with water and transferred to a 0.05% glucose medium for 3 h. Protein interaction was estimated using the yeast two-hybrid system, by measuring the β-galactosidase activity. Values correspond to means from 4 to 6 different transformants (bars indicate standard deviation), ***P < 0.001. (B) Yeast FY250 wild-type, gac1Δ and snf1Δ mutant strains containing the reporter plasmid pSH18-18 (6lexAop-lacZ) were transformed with plasmids pEG202-laforin and pACT2-malin. Transformants were grown until exponential phase (A600 0.5) in selective SC medium containing 4% glucose, and then washed with water and transferred to a 0.05% glucose medium for 3 h. Protein interaction was estimated using the yeast two-hybrid system, by measuring the β-galactosidase activity. Values represent means of 4 to 6 different transformants (bars indicate standard deviation), ***P < 0.001. (C) Yeast CTY10-5d strain containing plasmids pEG202-laforin and pACT2-malin was transformed with plasmids pSK93 (empty) or pSK-KDT172D, expressing a constitutively active form of AMPKα2 subunit. Transformants were grown until exponential phase (A600 0.5) in selective SC medium containing 4% glucose. Protein interaction was estimated using the yeast two-hybrid system, by measuring the β-galactosidase activity. Values represent means of 4 to 6 different transformants (bars indicate standard deviation), ***P < 0.001.
interaction between laforin and malin is regulated by AMPK, we postulated that the disruption of the endogenous laforin–malin complex is by expressing a dominant negative form of AMPK (α1-D157A; DN-AMPK) should result in an enhancement in the glycogenic activity of PTG. In agreement with this idea co-infection of FTO2B fibroblasts with Ad–GFP–PTG and Ad–DN-AMPK adenovirus resulted in a statistically significant enhancement of the glycogenic activity of PTG (Fig. 6A), as compared to the cells that were not infected with Ad–DN-AMPK, suggesting that the DN-AMPK prevented the downregulatory action of the laforin–malin complex, likely by interfering with the laforin–malin interaction. In this sense, western blot analysis indicated a partial recovery of the levels of GFP–PTG in the cells co-infected with the four adenoviruses (Fig. 6C). Interestingly, the levels of laforin were also higher in this case (Fig. 6C), perhaps as a consequence of the impairment of the interaction between laforin and malin.

We suggested above that the increase in the glycogenic properties of PTG produced by the co-infection with Ad–DN-AMPK (Fig. 6A) was due to the disruption of the endogenous laforin–malin complex. If our hypothesis was correct, elimination of laforin or malin from the cells should prevent the DN-AMPK-mediated enhancement of the glycogenic activity of PTG. To address this point, we used two primary fibroblast cell lines derived from LD patients carrying the laforin mutations Y86X and R241X, respectively, and cell lines from healthy control fibroblasts. These fibroblasts were infected with Ad–GFP–PTG and/or Ad–laforin and Ad–malin adenovirus. Figure 7A illustrates an enhanced accumulation of glycogen in all fibroblast cell lines when they were infected with Ad–GFP–PTG, which was higher in LD-derived fibroblasts. However, in contrast to healthy control fibroblasts, the co-infection with Ad–DN-AMPK of LD-derived fibroblasts did not enhance the glycogenic activity of PTG. These results suggest that in the absence of functional laforin–malin complex (either because one of the components is missing or because the formation of the functional complex is prevented), the glycogenic activity of PTG is at maximum.

**DISCUSSION**

LD is caused by mutations in the **EPM2A** or **EPM2B** genes, encoding laforin or malin, respectively. Although the roles of these two proteins in cellular physiology are still poorly understood, several reports have provide evidence suggesting that the disruption of protein–protein interactions involving laforin and malin are critical for the pathogenesis of LD. One of the histological determinants characteristic of LD is the accumulation of glycogen-like intracellular inclusions named Lafora bodies. Glycogen metabolism is mainly regulated by the phosphorylation of the proteins involved in glycogen synthesis (glycogen synthase, GS) and degradation (glycogen phosphorylase, Ph and glycogen phosphorylase kinase, PhK) (43,44). Interestingly, while there are several kinases (AMPK, PKA, CKI, GSK3, etc.) that inhibit glycogen
**Figure 6.** DN-AMPK prevents the effect of the laforin–malin complex on PTG. (A) Rat hepatoma FTO2B cells were infected with Ad–GFP–PTG (300 μl) or Ad–GFP (30 μl) and Ad–DN-AMPK (300 μl). Twenty-four hours after the infection, an aliquot of Ad–GFP–PTG infected cells was treated with AICAR (0.5 mM, 6 h) and the amount of glycogen in all the samples was determined as described in Materials and Methods. Bars indicate standard deviation of five independent experiments; ***P < 0.001. (B) FTO2B cells were infected with Ad–GFP–PTG (300 μl) and also with either Ad–GFP (30 μl), a combination of Ad–laforin and Ad–malin (100 μl each) or a combination of Ad–laforin, Ad–malin (100 μl each) and Ad–DN-AMPK (300 μl). Twenty-four hours after the infection, the amount of glycogen was determined as described in Materials and Methods. Bars indicate standard deviation of five independent experiments; ***P < 0.001. (C) Cell extracts (60 μg) from FTO2B cells treated as in section (B) were obtained and analyzed by western blotting using anti-GFP, anti-laforin, anti-HA (Ad–malin adenovirus produces an N-terminal HA-tagged malin), anti-AMPKα anti-GS or anti-actin antibodies.
synthesis by the phosphorylation of GS, there is only one known phosphatase, PP1, that induces glycogen synthesis by activating GS and inactivating the glycogen degradation enzymes Ph and PhK (43,44). PP1 is recruited to glycogen by a family of glycogen targeting proteins including GM, GL, PTG and R6 (43–45), whose overexpression results in glycogen accumulation (20,45). In this study, we present evidence for the critical role of the interaction between laforin, malin and PTG in LD pathogenesis.

We show here that laforin and malin play a crucial role in the regulation of glycogen biosynthesis in FTO2B hepatoma cells. In these cells, the laforin–malin complex counteracts the glycogenic effect of PTG because it promotes its ubiquitination and degradation. It has been described that in this type of cells PTG preferentially affects Ph and PhK over GS (46–48). Therefore, the laforin–malin dependent inactivation of PTG may ensure that Ph and PhK remain phosphorylated (active), which would prevent glycogen accumulation (Fig. 8). This mechanism is analogous to the one recently described in neurons (30). However, in neuronal cells, where no Ph and PhK are present but GS is clearly expressed (30,49), the role of the laforin–malin complex may be critical to maintain glycogen synthesis silenced in a cell that does not have the ability to degrade glycogen. LD patients lacking a functional laforin–malin complex would be unable to regulate PTG and GS, leading to glycogen accumulation in neurons (Fig. 8). Consistent with this interpretation, an LD patient with mutations in laforin shows a dramatic increase in the total levels of GS in skeletal muscle compared to a control individual (SRdeC, unpublished data). Further studies would be needed to reconcile these data with early studies in LD patients (50) and studies in mouse models (25,51) reporting that the activity of the enzymes involved in glycogen metabolism is not markedly affected.

Since the role of the laforin–malin complex is critical, we hypothesized that the formation of the laforin–malin complex must be also tightly regulated. Here, we provide evidence indicating that the formation of the laforin–malin complex is positively regulated by AMPK. We show that laforin, but not malin, can interact physically with the catalytic subunit of AMPK and that purified AMPK phosphorylates GST::laforin in vitro. Moreover, we demonstrate that the addition of a dominant negative form of the catalytic subunit of AMPK (DN-AMPK) prevents the function of the laforin–malin complex on the glycogenic activity of PTG, probably by interfering with the interaction between laforin and malin. As a result of this interference, malin is no longer able to access its substrates, laforin and PTG, thus the degradation of these two proteins is prevented.

These data provide evidence for an additional function of AMPK in glycogen metabolism, where its activation is known to lead to an increase in the phosphorylation and inactivation of GS and also to an increase in glucose uptake (52–54). However, in FTO2B hepatoma cells, the contribution of these two mechanisms to the overall regulation of glycogen accumulation seems to be fairly low since treatment of these cells with AICAR did not change the amount of glycogen accumulated in comparison with untreated Ad–GFP–PTG-infected cells. These results suggest that in these cells, the regulation of the function of PTG is key to adjust glycogen accumulation. A diagram depicting our hypothesis with the potential roles of the laforin–malin complex, its relationship with other proteins in glycogen metabolism and the differences between the mechanism operating in neurons and FTO2B hepatoma cells is provided in Figure 8.
Recently, an alternative function of laforin on glycogen homeostasis has been described (26). In this case, laforin acts as a phosphatase of complex carbohydrates (i.e. amylopectin) and it has been proposed that this function might be necessary for the maintenance of normal cellular glycogen.

In addition to PTG, malin and AMPK, laforin has been shown to interact with other proteins (16–19,55), suggesting that there are other regulatory roles for the laforin–malin complex besides glycogen metabolism. This is an important issue because it is currently unknown whether lafora bodies have a causative relationship with the epilepsy and neurodegeneration, or whether these LD features are independent consequences that result from the disturbance of a common physiological pathway. In this sense, it has been recently described that defects in protein degradation and clearance are likely to be the primary trigger in the pathophysiology of LD (56). Further elucidation of the mechanisms by which the formation of the laforin–malin complexes is regulated and of the mechanisms by which these complexes regulate PTG and glycogen synthesis in general, should lead to significant advances in the understanding of the pathogenesis of LD and hopefully, to the development of therapies.

MATERIALS AND METHODS
Recombinant plasmids
pGBT9-laforin and pACT2-laforin plasmids have been described previously (19). Plasmids pEG202-laforin and pGEX6P1-laforin were obtained by subcloning a BamHI–SalI fragment from pGBT9-laforin into pEG202 (Clontech) and pGEX6P1 (Amersham Biosciences), respectively. Malin was amplified from human genomic DNA by PCR and cloned into the prokaryote vector pGEX-A (Invitrogen). The final construct, pGST::malin, encoded a recombinant malin protein with GST fused at its N-terminus. Malin cDNA was also cloned into pcDNA3-HA (Invitrogen) and the yeast vectors pEG202 and pACT2 (Clontech). Laforin and malin containing plasmids were also used as templates for the introduction of EPM2A and EPM2B missense and non-sense mutations by PCR, using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) and appropriate mutagenic oligonucleotides. All expression constructs were fully sequenced to exclude the presence of undesired mutations resulting from PCR amplification. A pCINeo::laforin plasmid was used to express laforin in COS7 or HEK293 cells. A constitutively active form of the kinase domain of α2 catalytic subunit of AMPK (KDT172D) was constructed as in Scott et al. (40). The fragment was subcloned into plasmid pSK93 (57) to obtain plasmid pSK–KDT172D (58). Wild-type laforin and C266S mutant cDNAs were also subcloned into plasmid pSK93 to obtain plasmids pSK-laforin and pSK-laforin C266S. Plasmids pACT2-AMPKα2, pACT2-AMPKβ2, pACT2-AMPKγ1 and pCMV-HA-AMPKα2 are described in (58).

Yeast two-hybrid analyses
Yeast CTY10.5d strain was co-transformed with pACT2-laforin and different pEG202-malin plasmids (wild-type and mutants).

β-Galactosidase activity was assayed in permeabilized cells and expressed in Miller Units as in (59). For the yeast two-hybrid analyses using pGBT9-laforin (wild-type and mutants) as bait, yeast strain AH109 (Clontech) was co-transformed with pACT2-malin plasmid. Transformants were analyzed as in (19).

Expression of recombinant proteins in Escherichia coli
Escherichia coli transformants harboring different GST-fusions were grown in 500 ml of LB/ampicillin. Transformants were grown at 37 °C until the absorbance at 600 nm reached a value of around 0.3. IPTG (isopropyl-β-D-thiogalactoside) was then added to a concentration of 0.1 mM, and cultures were maintained overnight at 25 °C. Cells were harvested and resuspended in 20 ml of sonication buffer [50 mM HEPES-NaOH pH 7.0, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 2 mM DTT, 2 mM PMSF and complete protease inhibitor cocktail (Roche)]. Cells were disrupted by sonication and the fusion proteins purified by passing the extracts through 1 ml bed volume of glutathione-sepharose columns (Amersham Biosciences). GST-fusion proteins were eluted from the column with 25 mM glutathione. Samples were stored at −80 °C.

Co-immunoprecipitation and GST pull-down analyses
Immunoprecipitations were performed using transfected human embryonic kidney HEK293 cells. To identify laforin–AMPKα2 complexes, subconfluent cultures of HEK293 cells growing in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% inactivated fetal bovine serum (FBS, Gibco) plus 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine, were co-transfected with 3 μg of pCINeo-laforin and 3 μg of pCMV-HA-AMPKα2, using the calcium phosphate protocol. Transfected cells were lysed in lysis buffer [50 mM Tris–HCl pH 7.5; 10 mM NaCl, 50 mM EDTA; 15% glycerol, 1% nonidet P-40 (NP-40), complete protease inhibitor cocktail (Roche), 1 mM PMSF, 50 μM NaF and 5 mM Na2P2O7]. Cells were lysed by successive rounds of freezing and thawing. Cell lysates were then centrifuged at 13,000×g for 15 min at 4 °C. Laforin–AMPKα2 complexes were immunoprecipitated from the supernatants (500 μg of total protein) with anti-HA monoclonal antibody. Western blots of the immunoprecipitates were probed with a monoclonal anti-laforin antibody and a sheep anti-mouse IgG conjugated to HRP. The HRP signal was detected by using the ECL plus western blotting detection system (Amersham Biosciences).

Immunoblotting
Sixty microgram of total protein from the soluble fraction of cell lysates prepared as above were analyzed by SDS-PAGE and western blotting using appropriate antibodies: rabbit polyclonal anti-GFP (Molecular Probes), rabbit polyclonal anti-GS (60), rabbit polyclonal anti-phospho Ser461 GS (Cell signaling), mouse monoclonal anti-laforin (19), rabbit polyclonal anti-LexA (Invitrogen), mouse monoclonal anti-HA (Sigma), rabbit polyclonal anti-actin (Sigma) and rabbit polyclonal anti-AMPK (Cell Signaling).
In vitro ubiquitination assay

Ubiquitination assays were carried out by mixing purified GST-recombinant proteins (full-length maltin: maltin-C26S and maltin-D146N), mammalian E1 (5 ng/μl; Biomol), one type of mammalian E2 [UbcH7 or UbcH5a, or inactive [C85A] UbcH5a (25 ng/μl, Affinity)] and ubiquitin (100 ng/μl; Sigma), in ubiquitination buffer (250 mM Tris–HCl pH 7.4, 12.5 mM MgCl2, 2.5 mM DTT and 10 mM ATP). Samples were incubated at 25°C for 1.5 h and reactions were stopped by boiling the mixtures in SDS–PAGE sample buffer for 10 min. Proteins were separated by SDS–PAGE and visualized by immunoblotting using anti-GST (Santa Cruz Biotechnology) and anti-ubiquitin (FK2, Biomol) monoclonal antibodies.

Analysis of in vivo ubiquitination of PTG

To study the in vivo ubiquitination of PTG, HEK293 cells were transfected with pCMV-myc-R5 and combinations of pCMV-Ubiqux6His (encoding a modified ubiquitin, tagged with six His residues; a gift from Dr M. Rodriguez, Proteomics Unit, CIC-BioGUNE, Vizcaya), pcDNA3-HA-malin or pClNeo::lafarin plasmids, using the Fugene HD reagent (Roche) according to the manufacturer’s instructions. After 36 h of transfection, cells were lysed in buffer A (6 M guanidinium–HCl, 0.1 M sodium phosphate, 0.1 M Tris–HCl, pH 8.0). Four milligram of protein of a clarified extract (12 000 g, 15 min) was incubated with a 100 μM sodium phosphate, 0.1 M Tris–HCl, pH 8.0). Four milligram of protein of a clarified extract (12 000 g, 15 min) was incubated with a 100 μM of a mixture of 20 m M HEPES-NaOH pH 7.0, 1 mM dithiothreitol, 10 mM MgCl2, 300 μM AMP and 100 μM of a mixture of γ-32P-ATP (3000 Ci/mmol) and cold ATP, following the manufacturer’s instructions (Upstate). The reaction was incubated at 30°C for 1 h and stopped by boiling the mixtures in sample buffer. Samples were analyzed by SDS–PAGE and autoradiography. Two hundred and fifty nanogram of GST-fusion proteins were analyzed by SDS–PAGE and stained with Coomassie blue.

AMPK in vitro phosphorylation assay

Fifty nanogram of purified GST-fusion proteins were phosphorylated with 50 μU of AMPK (Upstate), in a final volume of 20 μl of a buffer containing 20 mM HEPES-NaOH pH 7.0, 1 mM dithiothreitol, 10 mM MgCl2, 300 μM AMP and 100 μM of a mixture of γ-32P-ATP (3000 Ci/mmol) and cold ATP, following the manufacturer’s instructions (Upstate). The reaction was incubated at 30°C for 1 h and stopped by boiling the mixtures in sample buffer. Samples were analyzed by SDS–PAGE and autoradiography. Two hundred and fifty nanogram of GST-fusion proteins were analyzed by SDS–PAGE and stained with Coomassie blue.

Adenovirus infection

Rat hepatoma FTO2B cells were cultured in complete Dulbecco’s Modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% inactivated fetal bovine serum (FBS, GIBCO), 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. Cells of number 10^6 were plated onto 60 mm-diameter culture dishes the day before infection. Infection with the corresponding adenovirus was carried out in 1 ml of complete DMEM containing 0.5% FBS. The following adenovirus were used in this work: Ad–GFP (10^11 pfu/ml), Ad–GFP–PTG (2x10^11 pfu/ml), Ad–lafarin (2x10^12 pfu/ml) and Ad–malin (4x10^12 pfu/ml) (30), and Ad–DN-AMPK (10^11 pfu/ml) kindly supplied by Dr Pascal Ferre, INSERM Unit 671, Universite Paris 6, Centre de Recherches Biomedicales des Cordeliers, Paris, France). Two hours after infection, adenovirus-containing medium was replaced with fresh complete DMEM containing 0.5% FBS. Twenty-four hours after infection, cells were washed with PBS and frozen in liquid N2 until analysis.

Primary fibroblasts from the skin of two LD patients, carrying the Y86X and R241X laforin mutations, were cultured using standard procedures and were routinely transformed using a plasmid (T22) containing the SV40 T antigen (kindly supplied by Dr M. Ugarte, Universidad Autónoma de Madrid, Spain). GM03349 fibroblasts from human skin (Coriell Cell Repositories, USA) were used as healthy controls. Fibroblasts were cultured in complete DMEM supplemented with 15% inactivated FBS. 150 000 cells were plated onto 60 mm-diameter culture dishes for 3 days. Fibroblasts were then infected with the corresponding adenoviruses as above.

Glycogen and GS activity determination

To measure glycogen content, cell monolayers were scrapped into 30% KOH and the extract was then heated at 100°C for 15 min. Glycogen was then measured as described previously (61). The amount of glycogen is expressed as the amount of released glucose per mg of total protein. GS activity was measured in cell homogenates in the absence or presence of 6.6 mM Glu-6P, as described previously (62). The ratio of glycogen synthase activity in the presence of 6.6 mM Glu-6P divided by the activity in the absence of Glu-6P is a non-linear measurement of the activation state of the enzyme. Values below 0.1 indicate an essentially fully inactive enzyme, whereas values above 0.7 are equivalent to full activation (63).

Statistical data analysis

Data are expressed as means ± standard deviation. Statistical significance of differences between the groups was evaluated by a paired Student’s t-test with two-tailed distribution. The significance has been considered at *P<0.05, **P<0.01 and ***P<0.001, as indicated in each case.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.
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