Laforin, defective in the progressive myoclonus epilepsy of Lafora type, is a dual-specificity phosphatase associated with polyribosomes

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The progressive myoclonus epilepsy of Lafora type is an autosomal recessive disorder caused by mutations in the EPM2A gene. EPM2A is predicted to encode a putative tyrosine phosphatase protein, named laforin, whose full sequence has not yet been reported. In order to understand the function of the EPM2A gene, we isolated a full-length cDNA, raised an antibody and characterized its protein product. The full-length clone predicts a 38 kDa laforin that was very close to the size detected in transfected cells. Recombinant laforin was able to hydrolyze phosphotyrosine as well as phosphoserine/threonine substrates, demonstrating that laforin is an active dual-specificity phosphatase. Biochemical, immunofluorescence and electron microscopic studies on the full-length laforin expressed in HeLa cells revealed that laforin is a cytoplasmic protein associated with polyribosomes, possibly through a conformation-dependent protein–protein interaction. We analyzed the intracellular targeting of two laforin mutants with missense mutations. Expression of both mutants resulted in ubiquitin-positive perinuclear aggregates suggesting that they were misfolded proteins targeted for degradation. Our results suggest that laforin is involved in translational regulation and that protein misfolding may be one of the molecular bases of the Lafora disease phenotype caused by missense mutations in the EPM2A gene.

INTRODUCTION

Progressive myoclonus epilepsy of Lafora type (LD; MIM 254780) is an autosomal recessive and fatal disorder with predominant central nervous system manifestations. LD patients develop stimulus-sensitive tonic-clonic, absence, drop and myoclonic seizures during adolescence, with a mean age at onset of ~15 years (1). After an initial period of normal development, rapid and progressive dementia follows, often with psychotic features. Patients finally become totally disabled and the average age at death is 20 years. Histological study of brain, heart and liver biopsies of patients show characteristic periodic acid–Schiff (PAS)-positive intracellular inclusions, called Lafora bodies. Since inclusion bodies were shown to be polyglucosan in nature, an enzyme defect in glycogen metabolism has been suggested to lead to the deposition of storage material and the disorder (2,3). The EPM2A gene, predicted to encode a putative protein tyrosine phosphatase, has recently been shown to be mutated in LD patients (4,5). To date, 4 microdeletions and 17 point mutations in EPM2A have been reported and all these mutations are expected to be loss-of-function mutations affecting the synthesis/function of laforin, the protein product of the EPM2A gene (4–6).

Laforin is a novel protein with a consensus sequence motif for protein tyrosine phosphatase but the complete protein sequence has not been reported. A full-length mouse homolog has been isolated that predicts a highly conserved mouse laforin (7). In order to understand the cellular functions of laforin and how its loss of function results in the LD phenotype, we cloned the full-length human EPM2A cDNA and studied the effect of missense mutations on laforin expression. In the present study, we show that the full-length laforin is an ~38 kDa dual-specificity phosphatase, primarily associated with polyribosomes. We also demonstrate that protein misfolding is one of the molecular bases of missense mutations in the EPM2A gene associated with the LD phenotype.

RESULTS

Cloning of full-length EPM2A cDNA

To isolate full-length cDNA for EPM2A, a human fetal brain cDNA library was probed with the partial sequence of EPM2A (see Materials and Methods). Six independent cDNA clones

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the 5′-untranslated region (5′-UTR) and first exon showed exact identity to the corresponding genomic sequence available for that gene. EPM2A, the coding region of full-length laforin 2b (GenBank accession no. AL023806). The newly defined 5′-ORF showed extended homology to the mouse Epm2a nucleotide and amino acid sequences (7). The similarity in size between the cDNA and signal seen on the northern analysis (~3.5 kb) (4, and data not shown) suggests that the full-length, or nearly full-length, cDNA was obtained. This EPM2A transcript is expected to code for 331 amino acids with a predicted molecular weight of 38 kDa and showed 89% identity and 94% similarity to the mouse homolog at the amino acid sequence level. In addition to the known protein tyrosine phosphatase domain, a search for a regulatory sequence prediction a weak candidate PEST sequence in the N-terminus of laforin (9; PEST search: http://www.icnet.uk/LRITu/projects/pest/runpest.html ). This 39 amino acid PEST sequence begins and ends with the positively charged arginine residues and had a PEST score of ~4.12 (Fig. 1).

Phosphatase activity

The protein encoded by EPM2A, named laforin, contains a consensus amino acid sequence suggestive of protein tyrosine phosphatase (4,5,7) (also see Fig. 1). To determine whether laforin is an enzymatically active protein tyrosine phosphatase, a bacterially expressed fusion protein was produced and the affinity purified glutathione S-transferase (GST) fusion protein was assayed for phosphatase activity against a synthetic phosphotyrosine analog, 3-O-methylfluorescein phosphate (OMFP). The usefulness of OMFP as a protein phosphatase substrate has been demonstrated previously and studies have shown that OMFP is an ideal substrate for dual-specificity phosphatases as they evoke faster kinetics for hydrolysis of OMFP compared with pNPP (10). Laforin showed phosphatase activity with OMFP in a dose-dependent manner, and arsename, the phosphatase inhibitor, inhibited this activity (Fig. 2A). To confirm that the phosphatase activity detected for GST–laforin was not due to Escherichia coli phosphatase contamination, site-directed mutation was made at the arginine 241 residue to create the most common natural mutation (Arg241stop) that encodes a truncated laforin lacking the phosphatase domain (4–6) (Fig. 1). The truncated GST–laforin fusion protein was produced and affinity purified glutathione transferase (GST)–laforin bacterially expressed fusion protein was produced and the protein tyrosine phosphatase or a dual-specificity phosphatase, phosphoserine/threonine and phosphotyrosine phosphatase activity that was partially inhibited by arsenate. Laforin is therefore an active protein tyrosine phosphatase (4,5,7) (also see Fig. 1). To determine whether laforin is an enzymatically active protein tyrosine phosphatase, a bacterially expressed fusion protein was produced and the affinity purified glutathione S-transferase (GST) fusion protein was assayed for phosphatase activity against a synthetic phosphotyrosine analog, 3-O-methylfluorescein phosphate (OMFP). The usefulness of OMFP as a protein phosphatase substrate has been demonstrated previously and studies have shown that OMFP is an ideal substrate for dual-specificity phosphatases as they evoke faster kinetics for hydrolysis of OMFP compared with pNPP (10). Laforin showed phosphatase activity with OMFP in a dose-dependent manner, and arsename, the phosphatase inhibitor, inhibited this activity (Fig. 2A). To confirm that the phosphatase activity detected for GST–laforin was not due to Escherichia coli phosphatase contamination, site-directed mutation was made at the arginine 241 residue to create the most common natural mutation (Arg241stop) that encodes a truncated laforin lacking the phosphatase domain (4–6) (Fig. 1). The truncated GST–laforin fusion protein was produced and affinity purified glutathione transferase (GST)–laforin bacterially expressed fusion protein was produced and the protein tyrosine phosphatase or a dual-specificity phosphatase, phosphoserine/threonine and phosphotyrosine phosphatase activity that was partially inhibited by arsename. Laforin is therefore an active dual-specificity phosphatase.

Expression of laforin in HeLa cells

The coding region of full-length EPM2A cDNA clone LDH1 was cloned into expression vectors and transfected into HeLa cells. For immunological detection of laforin protein, a polyclonal antibody was raised against a synthetic peptide. The anti-lafortin 2b antiserum (see Materials and Methods) detected a discrete ~38 kDa band in immunoblots of proteins from HeLa cells. For immunological detection of laforin protein, a polyclonal antibody was raised against a synthetic peptide. The anti-lafortin 2b antiserum (see Materials and Methods) detected a discrete ~38 kDa band in immunoblots of proteins from HeLa cells.
cells transfected with pcDNA-LDH construct (Fig. 3A). This molecular weight was very close to the predicted size, excluding the possibility of major post-translational modification of laforin in HeLa cells. Specificity and sensitivity of the antiserum were verified by probing the protein extracts of HeLa cells transfected with pcDNA-LDH+His or pEGFP-LDH expression constructs. Anti-laforin 2b antiserum detected the expected ∼40 and 65 kDa bands for the His-tagged and green fluorescent protein (GFP)-tagged laforin, respectively (Fig. 3A). No signal was observed in protein extracts from pcDNA or pEGFP vector transfected cells (Fig. 3A).

Reprobing the blot with anti-His or anti-GFP antibodies detected the respective bands with almost comparable intensity (data not shown; also see Fig. 4A–C). To check whether laforin is a soluble or membrane-bound protein, homogenates of transiently transfected HeLa cells were separated into soluble and membrane fractions by ultracentrifugation. Both the fractions were then resolved by SDS–PAGE and immunoblotting analysis revealed that laforin was mainly localized in the membrane fraction (Fig. 3B). Under our experimental conditions, endogenous laforin was undetectable in non-transfected cells (Fig. 3B). No reactivity was observed when the blots were reacted with the pre-immune serum (data not shown).

For subcellular localization of laforin, HeLa cells were transfected with either pcDNA-LDH (laforin alone), pcDNA-LDH+His (laforin with C-terminal Myc/His tag) or pEGFP-LDH (laforin with C-terminal GFP) expression constructs. Confocal imaging of laforin expression for all three constructs revealed a punctate cytoplasmic staining with much of the signal localized in the perinuclear region (Fig. 4A and D–F). Specificity of the anti-laforin 2b antiserum was confirmed by double staining the His-tagged laforin with anti-His antibody which showed complete overlap (Fig. 4A–C). Apparently, there was no difference in the subcellular localization of laforin having the C-terminal Myc/His or GFP tags (Fig. 4D–F). No staining was detected for anti-laforin or anti-His antibodies in cells transfected with pcDNA vector only and green fluorescence was detected predominantly in the nucleus of cells expressing GFP only (Fig. 4G). Taken together, these results suggest that laforin is a cytoplasmic protein and C-terminal addition of GFP or Myc/His tag does not alter its subcellular localization. Therefore, subsequent studies were done with cells expressing either Myc/His- or GFP-tagged laforin as it allowed flexibility in antibody combination for double staining.

From the staining pattern and its partition into the membrane fraction, it appears that laforin may associate with some cell organelles. Double staining was therefore performed with anti-laforin antibodies targeting specific cell organelles. No significant overlap was observed when laforin was co-stained with markers for mitochondria, lysosome, endosome or Golgi (data not shown except for lysosome: Fig. 5M–O). However, endoplasmic reticulum (ER) marker GRP94 showed extended overlap.
suggesting that laforin is associated with ER and that the extent of this overlap was similar between pcDNA-LDH+His- and pEGFP-LDH-expressing cells (Fig. 5A–F). Although the majority of the laforin staining was covered by GRP94, a considerable area (<30%), mainly in the cytoplasmic periphery, did not overlap with the ER marker. Since this region could possibly represent the ribosomal complex, laforin-expressing cells were double stained for the polyribosomal marker, QM protein (11,12). Staining of HeLa cells with QM protein showed a very similar punctate pattern and its double staining with laforin revealed its almost complete overlap (Fig. 5G–L). Thus, correlation of the laforin signal, partly with GRP94 and entirely with QM protein, suggested that laforin is primarily localized in polyribosomes associated with rough ER.

Localization of laforin in fractionated subcellular compartments

To confirm the results obtained from confocal microscopy that laforin is a ribosome-associated protein, pcDNA-LDH+His or pEGFP-LDH construct transfected cells were processed for cellular fractionation by differential centrifugation. Immunoblotting of the fractionated samples revealed that laforin protein co-localizes with the light membrane fraction enriched in polyribosomes and ER (Fig. 6A). Laforin-GFP fusion protein was also found in microsomal fraction confirming the confocal staining observations. As expected, QM protein (polyribosomal) and GAPDH (cytosolic) were mainly located in the light membrane and soluble fractions, respectively (Fig. 6A). Direct evidence for the association of laforin with ribosomes was obtained by purifying ribosomes through discontinuous sucrose gradient centrifugation. As shown in Figure 6B, laforin was detected in the ribosome fraction which also sedimented QM protein. Next, we determined whether association of laforin is restricted to polyribosomes or is also found in single ribosomes by analyzing the transiently transfected HeLa cell cytoplasmic fractions on linear 15–45% sucrose gradients. Immunoblot analysis of individual collected fractions revealed that laforin co-sedimented with polyribosomal fractions, whereas the fractions that contain single ribosomes were devoid of any signal (Fig. 6C).

Having established that laforin is localized in the polyribosomal fractions, we sought to clarify its mode of attachment. Laforin partitioned entirely into the aqueous phase when subjected to Triton X-114 phase separation, suggesting that it is not an integral membrane protein (Fig. 7). Sodium chloride and sodium carbonate treatments did not affect its ribosome association. On the other hand, partial extraction of laforin by 2.5 M urea indicated that its association may be through conformation-dependent, protein–protein interaction (Fig. 7).

Immunoelectron microscopic analysis of fractionated samples

Association of laforin with polysomes was confirmed at the ultrastructural level by immunogold labeling of light membrane fraction enriched in microsomes. In the majority of the samples, 5 nm gold particles were observed over 10 nm structures that can be judged morphologically as ribosomes (Fig. 8). No signal was observed on the membranous structure, most probably ER, associated with polysomes (Fig. 8A).

Subcellular distribution of laforin mutants in transfected HeLa cells

In addition to large deletions and nonsense mutations, a number of missense mutations have been identified in the EPM2A gene (4,5). Since missense mutations could cause a structural alteration in laforin, leading to impaired intracellular targeting, we analyzed the effect of two natural mutants,
Arg171His and Gln293Leu, reported previously as pathogenic alleles in LD families (4,5). Mutagenesis of the pcDNA-LDH+His construct was carried out in vitro and sequence-confirmed mutant constructs were transiently transfected into HeLa cells. In contrast to the punctate distribution of wild-type laforin, in the majority of transfected cells (>90%) both mutant proteins produced large vacuole-like structures in the perinuclear region of the cytoplasm (Fig. 9). These aggregates can be detected using either the N-terminal anti-laforin antibody or the C-terminal anti-His antibody, suggesting that they were full-length proteins. Double staining for ER, ribosomal and lysosomal markers revealed that these structures did not overlap with ER or ribosomes but that the majority of the aggregates were weakly positive for the lysosomal marker cathepsin-D (Fig. 9G–L). Strikingly, all the aggregates were ubiquitin positive suggesting that they were misfolded proteins targeted for degradation (Fig. 9M–R). Under our experimental conditions, such ubiquitin-positive abnormal structures were not found in non-transfected HeLa cells or in cells expressing wild-type laforin.

Figure 5. Co-localization of laforin with markers for ER and polyribosomes. HeLa cells that had been transiently transfected with pcDNA-LDH+His (A, G and M) or pEGFP-LDH (D and J) construct were double stained with anti-GRP94 (ER marker), anti-QM protein (polyribosome marker) or anti-cathepsin-D (lysosomal marker) antibody as indicated. Anti-His antibody was used to detect laforin in the pcDNA-LDH+His transfected cells. In (A)–(L), green (laforin) and red (respective marker) signals overlap, indicating partial and almost complete co-localization of laforin with ER and polyribosome markers, respectively. Absence of significant overlap between laforin (red) and lysosomal marker cathepsin-D (green) is evident in (M)–(O). Scale bar, 10 µm.
DISCUSSION

In order to understand the function of laforin and its involvement in LD, we isolated the full-length transcript of EPM2A and characterized its protein product. Sequence analysis of the full-length laforin predicted a weak PEST motif at the N-terminus of the protein. PEST sequences have been thought to be signals for proteolytic cleavage and were identified in several protein tyrosine phosphatases (13–16). Laforin contains the consensus active site of tyrosine phosphatases and has low level homology to active site of tyrosine phosphatases (17,20). Laforin-like proteins have not yet been identified in other classes although laforin appears to be conserved within mammals (7).

One of the primary objectives of the present study was to determine the subcellular distribution of laforin. We raised a polyclonal antibody against laforin and evaluated its cellular localization. The molecular weight of full-length laforin produced by prokaryotic and eukaryotic cells was very close to the predicted size suggesting that no major post-translational modification of laforin occurred in HeLa cells. Three different approaches clearly showed that the laforin is localized in the polysomes. In indirect immunofluorescent staining, the polysome marker QM protein showed almost complete overlap with laforin. QM is a conserved protein peripherally associated with the ribosome in the rough ER and has been suggested to be involved in 60S subunit assembly (11,12,21). Subcellular fractionation analysis showed that laforin was present in the light membrane fraction enriched in polysomes and was detected in the purified ribosomal fraction. Furthermore, electron microscopic observation of the microsomal fraction demonstrates that laforin is predominantly found over polysomes. Laforin was not detected in fractions that contain free ribosomes during sucrose gradient centrifugation, suggesting that distribution of laforin is restricted to polysomes. The requirement of 2.5 M urea for membrane disso-
absence of laforin might affect synaptic function, dramatically altering the function of neurons leading to epileptic seizures and neurodegeneration.

One of the characteristic features of LD is the formation of intracellular inclusions. These inclusions, called Lafora bodies, are not limited to the nervous system but are also observed in the heart, liver and skin (3,29,30). Lafora bodies are aggregates of linear long-chain glucose polymers, enzymatically digested by \( \alpha \)-amylase (3,29). LD has long been suspected to be a generalized storage disorder related to glycogenesis (2,29).

Glycogen synthase is the rate-limiting enzyme of glycogen deposition and is regulated by the dual-specificity kinase, glycogen synthase kinase-3 (GSK-3), which is also a polyribosome-binding protein (26,31). One of the upstream regulators of GSK-3 is p90 ribosomal kinase (26). It is tempting to speculate that GSK-3 is downregulated by laforin and its loss of function or reduced activity could lead to the production of excessive/abnormal glycogen and its aggregation as Lafora bodies.

Although neuronal cell death could be attributed to the presence Lafora bodies, it is unclear at present whether epilepsy is a consequence of neurodegeneration or caused directly by defective laforin. Further studies on in vivo models should help to elucidate the role of Lafora bodies in epilepsy and the function of laforin.

Various types of mutation (deletion, missense, nonsense and frameshifts) have been reported for EPM2A and yet the disease phenotypes of those patients were almost similar (4–6). All these mutations in EPM2A were predicted to be loss-of-function mutations. Although the absence (due to deletion) or truncation (nonsense mutations) of laforin may lead to lack of phosphatase activity, results presented here suggest that the molecular basis of some of the missense mutations in EPM2A is the inability of the laforin mutant to reach its final cellular destination. In the majority of cells, laforin mutants were found outside the ER and they could be detected using N- and C-terminal antibodies, suggesting that it is a full-length protein but not associated with polyribosomes. It has been shown recently that folding mutants of CFTR protein form perinuclear aggregates that are degraded by the cytoplasmic ubiquitin–proteasome pathway (32,33). In our study, expression of laforin mutants with missense mutations Arg171His and Glu293Leu resulted in ubiquitin-positive perinuclear aggregates suggesting that they were misfolded proteins targeted for degradation using a similar pathway. The presence of laforin mutants outside the polsosome complex is in agreement with the recessive nature of LD. We assume that laforin mutants become inactive in the ubiquitin complex and as a consequence the wild-type protein localized in the polysome is able to function continually in heterozygous cells. Our conclusion does not necessarily mean that this is the molecular basis of all missense mutations found in EPM2A. It seems very likely that some other mutations may have a different effect on laforin, for example its phosphatase activity or laforin’s half-life. In fact, under similar conditions, protein products could not be detected for two other missense mutant constructs (Ser25Pro and Arg108Cys; data not shown) and further studies are necessary to reveal their effects on laforin expression.

Although the enzymatic activity and subcellular localization of laforin were characterized in the present study, the precise function of laforin in cellular physiology remains largely unknown. It is interesting to note that there is yet another locus for LD that results in a clinically identical disease phenotype to

Figure 8. Electron microscopy demonstrates association of laforin with polysomes. In morphological observation of light membrane fractions incubated with monoclonal anti-His antibody, 5 nm gold particles (arrows) were evident over ribosome units. Lack of staining on the membrane associated with polysomes is evident in (A) (arrowheads). The small bar near the arrow in (B) measures the size of a ribosomal unit (≈10 nm). Scale bar, 50 nm.
that of individuals who had mutations in *EPM2A* (34). As this yet unknown gene product could act in the same metabolic pathway as laforin (5), further studies on proteins that interact with laforin might pave the way for understanding the specific role of laforin as well as in identifying the other disease loci.

**MATERIALS AND METHODS**

**Screening of cDNA library**

Based on the partial cDNA sequence reported for *EPM2A* (4), PCR-amplified exons 2, 3 and 4 were used to screen a human fetal brain cDNA library (UNI-ZAP XR insertion vector; Stratagene, La Jolla, CA) (35) using the standard method reported earlier (7). Approximately $2 \times 10^6$ plaques were hybridized overnight and positive plaques were transformed into plasmid by *in vivo* excision. The nucleotide sequence was determined by the dye-terminator method using ABI PRISM Autosequencer type 377 (PE Applied Biosystems, Foster City, CA).

**Construction of expression vectors and *in vitro* mutagenesis**

Mammalian expression vectors pcDNA3.1(+)/Myc-His B (Invitrogen, Carlsbad, CA) and pEGFPN1 (Clontech, Palo Alto, CA), and bacterial expression vector pGEX6P2 (Amersham Pharmacia Biotech, Piscataway, NJ) were used for expression studies. For all the constructs, the full-length *EPM2A* cDNA clone, LDH1, was used as template to PCR amplify the complete coding region. A forward primer (5′-ttcggatcccgctttggggtggtgcca-3′) with a modified 5′ end containing a *BamHI* site (bold) and a reverse primer (5′-acgctcgagtacaggctacacacagaagaacgaac-3′) with a 5′ *XhoI* site (bold) were used for amplification. Following *BamHI* digestion, the fragment was ligated into the *BamHI* and *SmaI* sites of pGEX6P2 vector. In this construct, called pGEX-LDH, the 5′ end of the *EPM2A* coding region was joined in-frame to encode a GST–laforin fusion protein. The same strategy was used to construct the mammalian expression construct except that the sequence of forward primer (5′-tgcagagacgacgtgttctc-3′) was derived from 157 bases upstream of the ATG start codon, a Kozak consensus translation signal sequence. The *XhoI* site of the reverse primer removed the stop codon; therefore, on *XhoI* digestion and ligation into the *BamHI* and *SmaI* sites of pGEX6P2 vector. In this construct, called pGEX-LDH, the 5′ end of the *EPM2A* coding region was joined in-frame to encode a GST–laforin fusion protein. The same strategy was used to construct the mammalian expression construct except that the sequence of forward primer (5′-tgcagagacgacgtgttctc-3′) was derived from 157 bases upstream of the ATG start codon, a Kozak consensus translation signal sequence. The *XhoI* site of the reverse primer removed the stop codon; therefore, on *XhoI* digestion and ligation into the *EcoRV–XhoI* double-digested pcDNA3.1(+)/Myc-His B vector, the coding region of *EPM2A* was maintained in-frame to add the Myc and His tag sequence at the C-terminus of laforin. This construct was named pcDNA-LDH+His. Another construct was created in which a stop codon was introduced between the laforin and Myc/His coding region by deleting the *XbaI* site (end-filling and ligation) of the pcDNA-LDH+His vector. This construct (called pcDNA-LDH) would code full-length laforin without the C-terminal Myc/His tag. For the pEGFPN1 construct, a *HindIII–SacI* fragment containing the coding region and 5′-UTR of *EPM2A* was excised from pcDNA-LDH and subcloned into the pEGFPN1 vector, creating the construct pEGFP-LDH. On transfection, pEGFP-LDH expressed laforin as a fusion protein to the N-terminus of enhanced green fluorescent protein (EGFP). The ORF of all constructs was verified by sequencing. Point mutations in the *EPM2A* coding region were

![Figure 9](image-url)

**Figure 9.** Distribution of Arg171His and Gln293Leu laforin mutants in transiently transfected HeLa cells as indicated. Double immunofluorescence staining was performed with anti-QM (polyribosome), anti-cathepsin D (lysosomal) or anti-ubiquitin antibodies. Laforin was stained with anti-His antibody. Note the formation of perinuclear vacuole-like structures that are partially overlapped with cathepsin D and ubiquitin immunostaining but not with the QM protein. Scale bar, 10 µm.
generated by using the Stratagene QuikChange site-directed mutagenesis kit according to the manufacturer’s instructions. Briefly, complementary primers containing the desired single base change were used in the PCR amplification of expression constructs. Following digestion with DpnI, the PCR products were used to transform E.coli XL1-Blue cells (Stratagene) and independent clones were isolated and mutations were confirmed by sequencing.

Expression, purification and phosphatase assay for GST–laforin fusion protein

Escherichia coli BL21 cells containing pGEX-6p, pGEX-LDH or pGEX-LDH-Arg241stop mutant constructs were cultured at 20°C for 16 h. Purification of expressed GST or GST–laforin fusion proteins were performed as described (36). Phosphatase activity of purified GST–laforin was measured at 37°C using 200 µM of OMFP (Sigma Aldrich, St Louis, MO) as a substrate in 500 µl of reaction buffer (100 mM sodium acetate, 1 mM EDTA, 1% DMSO; pH 8.0). After incubation for 1 h, the increase in absorbance at 477 nm of the 3-O-methylfluorescein product measured. Protein tyrosine and protein serine/threonine phosphatase activities of laforin were measured at 30°C for 8 h using 32P-labeled myelin basic protein (MyBP) as a substrate in 50 µl of reaction solution. 32P-labeled MyBP was prepared by in vitro phosphorylation of serine and threonine residues with cAMP-dependent protein kinase (New England Biolabs, Beverly, MA) or phosphorylation of tyrosine residues with c-abl (Calbiochem-Novabiochem, San Diego, CA) or phosphorylation of tyrosine residues with c-abl (Calbiochem-Novabiochem, San Diego, CA) or phosphorylation of tyrosine residues with c-abl (Calbiochem-Novabiochem, San Diego, CA) or phosphorylation of tyrosine residues with c-abl (Calbiochem-Novabiochem, San Diego, CA) or phosphorylation of tyrosine residues with c-abl (Calbiochem-Novabiochem, San Diego, CA).

For the preparation of anti-laforin antibody, a synthetic peptide corresponding to amino acid residues 86–101 (YKFLKREP-GGELSWEG; deduced from the full-length cDNA clone LDH1) with one cysteine residue added at the C-terminus of the peptide. For antibody production, the peptide was coupled with keyhole limpet hemocyanin. This conjugate was mixed with Freund’s complete adjuvant in phosphate-buffered saline (PBS) and injected into two rabbits at a dose of 1 mg protein/injection. Rabbits were boosted with peptide sequence mixed with incomplete adjuvant in PBS at 3 and 6 weeks after the first injection and bled at 8 weeks. Antiserum obtained from these rabbits was called anti-laforin 2b.

Commercial antibodies used and their source

The primary antibodies used in the present study include mouse monoclonal anti-His (C-term)inus) (His tag antibody; Invitrogen), rabbit polyclonal anti-human cathepsin D (Lysosomal marker; Upstate Biotechnology, Lake Placid, NY), goat polyclonal anti-GRP94 (ER marker; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-ubiquitin (Dako, Copenhagen, Denmark), rabbit polyclonal anti-QM protein (C-17; Santa Cruz Biotechnology) and mouse monoclonal anti-GAPDH (Chemicon-Novabiochem, Temecula, CA). Secondary antibodies used in immunofluorescence microscopy were anti-mouse IgG–rodamine conjugate (Molecular Probes, Eugene, OR), anti-goat rhodamine conjugate (Molecular Probes), Alexa Fluor anti-rabbit IgG–FITC conjugate (Molecular Probes) and Alexa Fluor anti-mouse IgG–FITC conjugate (Molecular Probes).

Confocal microscopy

Transiently transfected HeLa cells were fixed with 2% paraformaldehyde in PBS for 15 min, and permeabilized with 0.1% Triton X-100 in PBS for 5 min. The cells were incubated with blocking solution (5% normal goat serum and 5% fish gelatin in PBS) for 30 min, and then incubated with primary antibody in blocking solution (1:100 dilution for anti-laforin 2b) for 1 h at room temperature. After thorough washing with PBS, the cells reacted with secondary antibody (1:1000 dilution) for 1 h at room temperature, washed three times in PBS and the fluorescence was observed with a confocal laser scanning microscope (Olympus Personal Confocal System; Olympus, Tokyo, Japan).

Cell fractionation and membrane extraction

Transiently transfected confluent HeLa cells were placed on ice and washed twice with ice-cold PBS. Cells were scraped from the dishes and homogenized in a hypotonic buffer (0.25 M sucrose, 10 mM Tris–HCl, 10 mM NaCl, 1 mM EDTA, pH 7.5) supplemented with a mixture of protease inhibitors (Boehringer Mannheim, Mannheim, Germany). The lysate was centrifuged at 3300 g for 5 min to remove nuclei and the supernatant was used as total cytosolic protein. Soluble and membrane protein fractions were prepared according to the protocol described by Miyawaki et al. (37). Briefly, the post-nuclear fraction was centrifuged again at 100 400 g for 60 min at 4°C in a Beckman TL100.4 rotor and separated into soluble (supernatant) and membrane (pellet) fractions. Subcellular fractionation by differential centrifugation was performed with transfected HeLa cell homogenates by progressively increasing the centrifugal force (38). At each step, the pellet was saved as a designated fraction and the supernatant was carried on to the next centrifugation step. The centrifugation steps were as follows: 600 g for 10 min to collect nuclei, 10000 g for 10 min to collect the heavy membrane fraction (mitochondria, lysosomes and peroxisomes), 100 400 g for 60 min, and the pellet and supernatant were used as the light membrane (microsomes) and cytoplasmic fractions, respectively. Ribosomes were prepared essentially as described earlier (39). After the removal of the heavy membrane fraction, the supernatant containing light membrane and soluble proteins was overlaid onto a discontinuous sucrose gradient of

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0.7 M sucrose on top of the 1.6 M sucrose. Both the sucrose solutions contain 20 mM Tris–HCl pH 7.4, 100 mM KCl and 5 mM MgCl₂. The sample was centrifuged at 300 000 g for 18 h at 4°C in a Beckman TL100.4 rotor and the pellet was saved as the ribosome fraction. For membrane extraction, the light membrane pellet was treated either with 1.5 M NaCl, 0.1 M Na₂CO₃ or 2.5 M urea. After being incubated on ice for 60 min, the suspensions were centrifuged at 100 400 g for 60 min to obtain the supernatants and membrane pellets. Triton X-114 phase separation of the membrane fraction was carried out following the method of Bordier (40).

**Sucrose density ultracentrifugation**

Cytosplasmic extracts, of 16 OD at 260 nm, from HeLa cells transiently transfected with pcDNA-LDH+His construct were analyzed by sedimentation velocity in a 15–45% linear sucrose gradient made up of 25 mM Tris–HCl pH 7.4, 100 mM KCl and 5 mM MgCl₂. After centrifugation at 38 000 r.p.m. at 4°C in a Beckman SW41Ti rotor for 2 h, fractions of gradients were obtained by upward displacement using an ISCO (Tokyo, Japan) density gradient fractionator (model 185) and absorbance was measured at 254 nm. For western analysis, fractions were diluted with 1 vol of buffer and particles were pelleted by centrifugation at 300 000 g for 2 h.

**Immunoblotting**

For western blot analysis, protein samples (50 µg) were run on a 15–25% gradient SDS–polyacrylamide gel and transferred onto a nitrocellulose filter (0.45 µm; Schleicher & Schuell, Dassel, Germany) using an electroblot apparatus (Bio-Rad, Hercules, CA) at 100 V for 1 h in transfer buffer [25 mM Tris–HCl, 192 mM glycine, 0.1% SDS, 20% (v/v) methanol]. The filter was incubated in blocking solution (50 mM Tris–HCl, 200 mM NaCl, 1 mM MgCl₂, pH 7.4) containing 3.5% non-fat dry milk powder for 1 h at 37°C. The membrane was processed through sequential incubations with primary antibody (anti-laforin, 1:500 dilution) for 1 h, and then with 0.4 µg/ml horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Immunoreactive proteins on the filter were visualized using the Renaissance Chemiluminescence kit (NEN Life Science Products, Boston, MA).

**Immunoelectron microscopy of fractionated samples**

For morphological observation light membrane fraction, samples were adsorbed onto a supporting membrane placed on 400-mesh grids. Grids were fixed by floating on 2% paraformaldehyde–2% glutaraldehyde in 0.1 M phosphate buffer for 5 min. After three washes with distilled water, grids were negatively stained with 2% sodium phosphotungstic acid and dried. Specimens were observed in an LEO 912AB electron microscope (LEO, Cambridge, UK). For immunostaining, grids with absorbed sample were prefixed in 2% paraformaldehyde and incubated with washing buffer (0.1 M Tris-buffered saline) containing 1% normal goat serum for 5 min. Grids were subsequently incubated with washing buffer containing mouse monoclonal anti-His antibody (1:500 dilution) for 15 min, washed three times with the washing buffer and then incubated 20 times with diluted secondary colloidal gold-conjugated antibody (5 nm; British BioCell International, Oxford, UK) for 15 min. Treated grids were fixed with 2% glutaraldehyde and processed for negative staining and observed as mentioned above.

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**REFERENCES**


