The malin–laforin complex suppresses the cellular toxicity of misfolded proteins by promoting their degradation through the ubiquitin–proteasome system

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Lafora disease (LD), a progressive form of inherited epilepsy, is associated with widespread neurodegeneration and the formation of polyglucosan bodies in the neurons. Laforin, a protein phosphatase, and malin, an E3 ubiquitin ligase, are two of the proteins that are defective in LD. We have shown recently that laforin and malin (referred together as LD proteins) are recruited to aggresome upon proteasomal blockade, possibly to clear misfolded proteins through the ubiquitin–proteasome system (UPS). Here we test this possibility using a variety of cytotoxic misfolded proteins, including the expanded polyglutamine protein, as potential substrates. Laforin and malin, together with Hsp70 as a functional complex, suppress the cellular toxicity of misfolded proteins, and all the three members of this complex are required for this function. Laforin and malin interact with misfolded proteins and promote their degradation through the UPS. LD proteins are recruited to the polyglutamine aggregates and reduce the frequency of aggregate-positive cells. Taken together, our results suggest that the malin–laforin complex is a novel player in the neuronal response to misfolded proteins and could be potential therapeutic targets for neurodegenerative disorders associated with cytotoxic proteins.

INTRODUCTION

Protein quality control is a stringent and critical feature of intracellular homeostasis. Irreversibly unfolded or misfolded proteins resulting from defects in the encoded proteins and/or proteasomal stress are rapidly degraded via the ubiquitin–proteasome system (UPS) (1,2). A striking pathological hallmark of many neurodegenerative disorders is the presence of ubiquitin-positive, intracellular inclusion bodies in affected regions of the brain (3). These inclusions, in the majority of such cases, are thought to be the aggregates of non-native neurotoxic proteins (hereafter referred to as ‘misfolded proteins’) that accumulate over time (2,3). Examples of toxic proteins include, but not limited to, polyglutamine proteins, polyalanine proteins and the mutant forms of α-synuclein and superoxide dismutase (2,3). Aggregates of these misfolded proteins are also known to recruit molecular chaperones and components of the UPS, thereby raising the possibility that an overload on the UPS is likely to promote the neurodegenerative process (2–4). This notion was strengthened by the findings that overexpression of chaperones and/or E3 ubiquitin–protein ligases ameliorated the cellular toxicity of misfolded proteins in cellular and animal models (5–9). Conversely, the inhibition of proteasome or defect in the UPS was shown to promote cellular toxicity by increasing the load of misfolded proteins in the cellular milieu (9,10). Thus, an imbalance between the capacity of UPS to clear toxic protein and the synthesis of aggregation-prone, misfolded proteins might initiate the onset and the progression of diseases symptoms (2,11).

UPS relies on the transfer of ubiquitin molecules to the target protein through three enzymatic steps, with the key steps of substrate selection and ubiquitin transfer being delegated to an enzyme called ubiquitin–protein ligase or

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the E3 enzyme (1). The complex process of UPS-mediated protein degradation is influenced by a large number of regulatory proteins, and E3 ligases offer substrate specificity to this process (1). Not surprisingly, therefore, mutations in genes involved in, or affecting, the UPS have been linked to several neurodegenerative diseases associated with protein misfolding (2). For example, Parkinson’s disease (PD) is caused by mutations in parkin, an E3 ubiquitin ligase, and UCH-L1, a deubiquitinating enzyme (12–14). Similarly, mutations in the NHLRC1 gene that encodes malin E3 ubiquitin ligase result in Lafora disease (LD), a neurodegenerative progressive epilepsy (15,16). Both malin and parkin are RING finger ubiquitin ligases, and the inhibition of proteasomal activity causes both proteins to form centrosomal aggresome that co-localizes with the UPS components (17,18). Aggresomes are thought to provide a subcellular locale for the E3 enzymes to ubiquitinate and degrade misfolded proteins when the cellular levels of the latter increase beyond a threshold (4,19). Corroborating this suggestion, the overexpression of parkin was shown to reduce the aggregation and cytotoxicity of ataxin-1-containing expanded polyglutamine repeats (8) and mutant α-synuclein (20). Intriguingly, expanded polyglutamine proteins and mutant α-synuclein are known to get recruited to the aggresome upon overexpression or when the proteasome is blocked (21–23). Thus, misfolded proteins that target aggresome are potential substrates for the aggresome-positive E3 ligases.

In addition to the NHLRC1 gene, LD may also be caused by defects in the gene EPM2A that codes for a dual-specificity protein phosphatase, named laforin (24,25). Laforin is also known to interact with malin and serves as one of its substrates (16,18). We have shown recently that both malin and laforin proteins, hereafter referred to as LD proteins, are recruited to aggresome in response to proteasomal impairment, suggesting that the LD proteins might work together in the UPS pathway (18). Because malin and laforin are recruited to the aggresome, we addressed the possibility of whether the LD proteins would promote the degradation of misfolded proteins and rescue cells from their cytotoxic effect. We show here that laforin and malin form a functional complex that facilitates the clearance of misfolded proteins from the cellular milieu.

RESULTS

Overexpression of LD proteins decreases the cellular toxicity of misfolded proteins in neuronal and non-neuronal cells

Expanded polyglutamine proteins, polyalanine proteins and α-synuclein mutants are known to form aggresome-like structures either on their own or when expressed in the presence of MG132, a proteasomal inhibitor (21–23). We therefore evaluated the truncated huntingtin protein with 97 glutamine repeats and tagged to green fluorescent protein (tHtt-Q97-GFP) [associated with polyglutamine disorders (reviewed in 2)], 20 alanine repeats tagged to the carboxyl terminal of GFP (GFP-A20) [polyalanine disorders (reviewed in 2)], 20 aspartic acid repeats tagged to the carboxyl terminal GFP (GFP-D20) [skeletal dysplasias (26)] or the GFP-tagged α-synuclein missense mutant A30P protein [PD (27)] as potential substrates for malin and laforin. As demonstrated earlier (21–23), the overexpression of each one of these proteins, hereafter referred to as misfolded proteins, resulted in the formation of perinuclear aggregates when the proteasome is blocked or otherwise (Supplementary Material, Figs S1 and S2). A construct that expresses GFP-fused to 20 repeats of glutamine residues (GFP-Q20) or the wild-type α-synuclein was used as the control (Supplementary Material, Fig. S1). One hundred percent of the cells that expressed tHtt-Q97-GFP, GFP-A20, GFP-D20, GFP-tagged α-synuclein mutant A30P protein or its wild-type form exhibited perinuclear aggregates upon MG132 treatment (Supplementary Material, Figs S1 and S2).

The transient expression of tHtt-Q97-GFP, GFP-A20, GFP-D20 or the α-synuclein A30P mutant protein has induced substantial cell death in COS-7 cells (Fig. 1A and B and Supplementary Material, Fig. S1). Intriguingly, the co-expression of wild-type malin or laforin significantly reduced the cell death caused by these misfolded proteins \( (P < 0.005) \) (Fig. 1A and B). For example, the toxicity of tHtt-Q97-GFP was brought down to 50% by malin or laforin when co-expressed. This protective effect, however, was not seen when the LD-associated mutant forms of malin or laforin or when β-galactosidase was co-expressed (Fig. 1A–C). We next explored whether the observed protective effect of LD proteins can also be replicated in neuronal cells. As shown in Figure 2A, the co-expression of LD proteins has significantly reduced the toxicity of each of the four misfolded proteins tested in the Neuro2A mouse neuroblastoma cell line, suggesting that laforin and/or malin proteins confer protection against toxic misfolded proteins regardless of the cell line used.

Partial knockdown of LD proteins enhances the toxicity of misfolded proteins in neuronal cells

We have also checked the effect of knockdown of endogenous laforin or malin on the toxicity conferred by the misfolded proteins in Neuro2A cells. The RNAi-mediated knockdown of EPM2A or the NHLRC1 gene was validated (see Supplementary Material, Fig. S4), and Neuro2A cells were co-transfected with tHtt-Q97-GFP with an shRNAi construct for the knockdown of laforin, malin or an empty vector. A significant decrease in the survival rate was observed when tHtt-Q97-GFP was co-expressed with the shRNAi construct for the knockdown of laforin, malin or an empty vector. However, loss of laforin or malin does not affect the survival of cells when they do not express tHtt-Q97-GFP (Fig. 2B). Similar results were obtained with the COS-7 cell line (data not shown).

Malin and laforin proteins suppress the toxicity of misfolded proteins by promoting their degradation

In order to check whether malin and laforin rescue cells from the toxicity of misfolded protein by enhancing their rate of degradation, we co-expressed tHtt-Q97-GFP with either the wild-type malin, laforin or their catalytic mutants and looked for the fold difference in the cellular levels of tHtt-Q97-GFP...
by immunoblot analysis by loading equal amount of total protein. A significant reduction in the cellular levels of tHtt-Q97-GFP was seen when the misfolded protein was expressed with the wild-type malin or laforin when compared with those that co-expressed the mutant forms or that were treated with a proteasomal inhibitor (Supplementary Material, Fig. S3B). Thus, malin and laforin likely facilitate the degradation of misfolded proteins through the proteasomal system. To further prove this point, we expressed tHtt-Q97-GFP with increasing concentrations of wild-type or the mutant forms of malin or laforin and checked for its cellular levels by the immunoblot analysis (Fig. 3). We show that the tHtt-Q97-GFP protein levels were decreased in a malin or laforin dose-dependent manner, and such an effect was not observed for the mutant form (compare the signal intensities of tHtt-Q97-GFP in Fig. 3A with B and C and Fig. 3D with E and F).

We next wanted to check whether LD proteins reduce the cellular levels of misfolded proteins by enhancing their rate of degradation. For this, we have used an inducible system, wherein the expression of tHtt-Q97-GFP could be modulated by ponasterone (28) (Fig. 4A). The inducible tHtt-Q97-GFP was expressed for 12 h in Neuro2A cells either alone or with the wild-type or the mutant forms of laforin/malin, and then processed for the pulse-chase analysis by withdrawing the ponasterone from the medium for various time points (Fig. 4B). As shown in Figure 4B and C, the overexpression of wild-type malin or laforin, but not their mutants, had significantly decreased the half-life of tHtt-Q97-GFP.

FIGURE 1. Bar diagrams showing levels of toxicity (percent cells showing apoptotic features) in COS-7 cells transiently transfected with expression constructs as indicated. The value of each bar represents the mean average of three independent transfections, with a minimum of 250 transfected cells scored for each transfection. Error bars indicate the SD of the mean. (A) Difference in the number of cells showing apoptotic features (cells in which the nucleus is fragmented into dense granular particles—see Supplementary Material, Fig. S1B and D) between the groups of cells that express only the misfolded protein when compared with groups that co-express the wild-type (WT) or the mutant forms of malin (C26S and NHL-del) (18) was calculated. (B) Difference in the number of cells showing apoptotic features between the groups of cells that express only the misfolded protein when compared with groups that co-express the wild-type (WT) or the mutant forms of laforin (W32G and Q293L) (18) was calculated. (C) Cytotoxicity of cells that co-express misfolded proteins (as indicated) and β-galactosidase was calculated. (D) Cytotoxicity of cells that express the GFP, GFP-Q20 or the wild-type α-synuclein (α-Syn-WT) is shown. The difference in the P-value less than 0.05 or 0.005 was denoted over the bar by a single asterisk or double asterisks (*), respectively, as derived from a paired t-test.

LD proteins need each other to suppress the cellular toxicity of misfolded proteins

We next examined whether malin would require laforin, and vice versa, to confer the protection against the toxicity of misfolded proteins. For this, we transiently co-expressed tHtt-Q97-GFP with malin and knocked down laforin or co-expressed tHtt-Q97-GFP with laforin, but knocked down malin in both COS-7 and Neuro2A cell lines (Fig. 5A and B). Control experiments were performed in parallel, wherein tHtt-Q97-GFP was co-expressed with empty knockdown vector and laforin or malin. As shown in Figure 5A and B, the overexpression of malin was not able to suppress the toxicity of tHtt-Q97-GFP when laforin was knocked down, and similar observations were made for laforin when malin was knocked down in both the cell lines tested. Thus, laforin and malin appear to function together as a complex, and the loss of anyone of them would make the other protein ineffective against misfolded proteins.

We next checked whether the LD proteins require each other for reducing the cellular levels of misfolded protein. For this, we co-expressed tHtt-Q97-GFP with the knockdown construct for laforin and increasing concentrations of wild-type malin (Fig. 5C). Conversely, the tHtt-Q97-GFP was expressed with the knockdown construct for malin and increasing concentrations of laforin protein (Fig. 5D). Malin was unable to reduce the cellular levels of tHtt-Q97-GFP when the expression of laforin was blocked (Fig. 5C). Similar observations were made for laforin when malin was knocked down (Fig. 5D). However, co-expression of empty shRNA vector did not affect the protective effect of laforin or malin on tHtt-Q97-GFP (Fig. 5E and F).

Malin and laforin physically interact with misfolded proteins

We have also examined whether malin and laforin promote the degradation of misfolded proteins by physically interacting with them. For this, we used tHtt-Q97-GFP and GFP-D20 as two distinct misfolded proteins and tested for their interaction with laforin or malin. As a control, we used an overexpression
construct for GFP. As shown in Figure 6A, the His-tagged malin and laforin were able to pull-down the tHtt-Q97-GFP protein. Laforin and malin were also able to pull-down GFP-D20, and no interaction was found with GFP, suggesting that the observed interaction with LD proteins is not limited to expanded polyglutamine protein but a generic response to a wide variety of misfolded proteins. As laforin and malin require each other to degrade the misfolded proteins, we next checked whether the observed interaction between misfolded protein and malin is independent of laforin (or vice versa) or not. For this, we have chosen GFP-D20 because of its robust expression and co-expressed it with laforin and knocked down malin using the shRNA construct. In parallel, GFP-D20 was expressed with malin, and laforin was knocked down (Fig. 6B). As a control, laforin or malin was co-expressed with GFP-D20 in the presence and absence of malin. However, malin was unable to pull GFP-D20 when laforin was knocked down. Taken together, these observations suggest that, within the malin–laforin complex, laforin is the one that makes direct interactions with the misfolded proteins.

Malin–laforin complex degrades misfolded proteins through the ubiquitin–proteasomal pathway

As malin is an E3 ubiquitin ligase and malin forms a complex with laforin, we wanted to check whether the malin–laforin complex promotes the degradation of the misfolded proteins through the UPS. For this, we have used a mutant ubiquitin, K48R, which is known to block the K48-linked polyubiquitination when overexpressed (29). We have used a construct that expresses wild-type ubiquitin as the control (Fig. 7A). As shown in Figure 7A, malin or laforin was unable to rescue the toxicity of tHtt-Q97-GFP when co-expressed with the ubiquitin mutant K48R, suggesting that the malin–laforin complex rescues the cells from the toxicity of misfolded proteins through polyubiquitination. In contrast, the co-expression of wild-type ubiquitin, as expected, did not alter the protective effect of laforin or malin. These observations were further strengthened by looking at the ubiquitinated forms of tHtt-Q97-GFP in pull-down assays. As shown in Figure 7B, the co-expression of wild-type malin or wild-type laforin resulted in the increased cellular levels of ubiquitinated tHtt-Q97-GFP when compared with cells that had co-expressed the mutant forms of malin/laforin or the empty vector. Similarly, ubiquitinated tHtt-Q97-GFP was not observed when the ubiquitin mutant K48R was co-expressed (Fig. 7B). These observations demonstrate that the LD proteins promote the degradation of misfolded protein via the polyubiquitination process.

The ubiquitin–proteasome and autophagy–lysosome pathways are the two major routes for the clearance of unwanted and misfolded toxic proteins in eukaryotic cells (3). We have therefore tested whether malin and laforin use, in addition to the UPS, the autophagy–lysosome pathway for the clearance of misfolded proteins. For this, COS-7 or Neuro2A cells were co-transfected with wild-type malin or laforin and tHtt-Q97-GFP and tested for cellular toxicity using a panel of inhibitors, a strategy that had been used previously for the study of polyglutamine and polyalanine proteins (30) (see Fig. 8 for details on inhibitors). Blocking
Malin and laforin interact with Hsp70 and all the three members of this complex are required for the suppression of cellular toxicity of misfolded proteins

As malin, with the help of laforin, appears to recognize a diverse set of proteins for the polyubiquitination process, we speculated that malin might interact with Hsp70 to target the misfolded proteins for proteasomal degradation. To test our hypothesis, we first examined, by cell death assay, whether knockdown of endogenous Hsp70 abrogates the protection against cell death conferred by malin or laforin. As shown in Figure 9A and B, malin or laforin was unable to rescue cell death promoted by tHtt-Q97-GFP when Hsp70 was knocked down. This observation demonstrates that Hsp70 is required for malin and laforin to impart the cytoprotective effect. Conversely, the overexpression of Hsp70 rescued the cells from the toxicity of tHtt-Q97-GFP at levels similar to that of wild-type malin or laforin, either on its own or when co-expressed with the wild-type malin or laforin (Fig. 9A and B). We have therefore checked whether Hsp70 would require malin or laforin for its cytoprotective function. For this, we transiently co-expressed Hsp70 and tHtt-Q97-GFP along with the knockdown shRNA construct for malin or laforin and scored for cell death (Fig. 9C). The control set, wherein Hsp70 and tHtt-Q97-GFP were co-expressed with an empty vector for shRNA, showed significant reduction in the toxicity of tHtt-Q97-GFP (Fig. 9C). In contrast, the overexpression of Hsp70 was not able to rescue the cells from the toxicity of tHtt-Q97-GFP when the expression laforin or malin was knocked down (Fig. 9C), suggesting that Hsp70 requires both laforin and malin to reduce the toxicity of misfolded proteins.

We next examined whether malin and laforin physically interact with Hsp70. As shown in Figure 9D, malin and laforin were able to pull-down Hsp70. As malin and laforin function as a complex, we next checked which of these two proteins interact with Hsp70. As shown in Figure 9E, laforin was able to pull-down Hsp70 even in the absence of malin, whereas malin was unable to pull Hsp70 when the expression of laforin was blocked, suggesting a direct interaction between Hsp70 and laforin. Finally, we have also checked whether Hsp70 is critical for the interaction between misfolded proteins and laforin/malin. As shown in Figure 9F, laforin was able to pull-down GFP-D20 even in the absence of Hsp70, whereas malin could not. Thus, the interaction of misfolded proteins with laforin appears to be independent of Hsp70, but for malin, it is Hsp70-dependent.

Malin–laforin complex associates with cellular aggregates of tHtt-Q97-GFP and facilitates their clearance

We checked whether malin and laforin would co-localize with tHtt-Q97-GFP aggregates and enhance their clearance. For this, COS-7 cells were co-transfected with tHtt-Q97-GFP and laforin or malin and processed for indirect immunofluorescence staining. Under normal cell culture conditions, malin localizes to both nucleus and cytoplasm in COS-7 cells, whereas the localization of laforin was restricted to the cytoplasm (18). When expressed with tHtt-Q97-GFP, however, the majority of the malin or laforin proteins targeted the tHtt-Q97-GFP aggregates and co-localized with them (Fig. 10A). We therefore reasoned that, when expressed together, the malin–laforin complex reduces the cellular toxicity of misfolded proteins by ‘dissolving’ the aggregates. We therefore transiently expressed tHtt-Q97-GFP alone or with the wild-type malin or laforin and scored for aggregate-positive cells with GFP expression (Fig. 10B). When expressed alone, 25% of the GFP-positive cells show cytoplasmic and/or nuclear aggregates. In the presence of wild-type malin or laforin, however, there was a significant decrease in the number of aggregate-positive cells (~8%) (Fig. 10B). In contrast, knockdown of laforin or malin had increased the frequency of cells having aggregates, but this difference was not statistically significant (Fig. 10B).
Consistent with these findings, the co-expression of laforin or malin has decreased the level of high-molecular-weight bands of the tHtt-Q97-GFP protein trapped in the stacking gel (Fig. 10C), suggesting that malin and laforin could bring down the toxicity by altering the aggregation property of the misfolded proteins.

Malin and laforin proteins are recruited to huntingtin aggregates in the brain of HD exon-1 transgenic mice

We have also explored whether malin and laforin are recruited to the neuronal inclusions in the R6/2 mice model for Huntington disease (31). Using double immunofluorescence staining, we show here that in the transgenic mice brain, malin and laforin proteins co-localize with the aggregates of mutant huntingtin (Fig. 11). Not all the huntingtin aggregates, however, were positive for the LD proteins. Fifty percent of the huntingtin aggregates in the cerebral cortex region stained positive for the anti-laforin or anti-malin antibody and their localization was much lower in cerebellum and hippocampus (~20%).

DISCUSSION

We have shown here that co-expression of malin or laforin clears misfolded toxic proteins through the ubiquitin–proteasome pathway and reduces their cellular toxicity. Although the present set of observations was made in cellular models under overexpression conditions, carefully chosen controls, as discussed below, suggest that the observed properties of LD proteins are not experimental artifacts. The experiments were performed in two different cell lines (COS-7 and Neuro2A), and identical observations were made, suggesting that the observed effects were not restricted to a given cell line. With regard to the toxicity conferred by the misfolded proteins, a significant reduction in the frequency of cell death was seen only when the wild-type malin or laforin was co-expressed. Neither their mutant forms nor did the β-galactosidase protein show any appreciable effect on the viability of cells co-expressing the misfolded proteins. This suggests that functional malin and laforin were required for the rescue and that the observed difference in the toxicity was not just due to the co-expression of any protein. Thirdly, partial knockdown of endogenous malin or laforin led to an increase in the toxicity of misfolded proteins. Finally, we show that laforin and malin co-localize with the mutant huntingtin aggregates in cellular and animal models.

Malin is known to ubiquitinate and promote the clearance of its substrates through proteasomal degradation (16,18,32–34). Consistent with this notion, we show here that the wild-type malin and wild-type laforin reduce the cellular levels of misfolded proteins in a dose-dependent manner, and this effect was abrogated when the cells were treated with a proteasomal inhibitor. A reduction in the cellular toxicity of misfolded proteins should therefore be due to their reduced cellular levels, as proteasomal inhibition leads to an increase in cell toxicity.
death frequency even in the presence of wild-type malin. This suggestion was strengthened by our observation that the co-expression of ubiquitin mutant K48R, suggesting that LD proteins clear misfolded proteins primarily through the UPS and via ubiquitination process, as shown in the ubiquitination assays. In addition to the UPS, misfolded proteins are known to be cleared through the autophagy–lysosome pathway (3). Indeed, there are reports that suggest that a given substrate could be either cleared through the UPS or the autophagy–lysosome system (3). The present study, involving specific blocker for the lysosome and autophagosome, suggests that malin and laforin use the UPS as the primary route for the clearance of misfolded proteins.

We found that malin and laforin proteins specifically bind to the misfolded proteins and promotes their ubiquitination. Moreover, the effect of malin and laforin on the rate of degradation of misfolded protein was dose-dependent; higher the level of malin or laforin, lesser the level of misfolded proteins. These findings suggest that the observed toxicity...
for the misfolded protein was not because of the saturation of the proteasomal function, but could perhaps be that the cellular machinery was unable to target the misfolded proteins to the proteasome by failing to ubiquitinate them. Although the role of malin is established as an E3 ubiquitin ligase, the specific function of laforin in UPS is not clear. It is likely that laforin acts as a 'substrate adaptor protein' to present the misfolded proteins to malin, because malin was unable to interact with the misfolded proteins when laforin was not present. Our observations that mutant laforin was unable to perform this function suggest that laforin might dephosphorylate some critical factors in the chaperone–misfolded protein complex, and this step is critical for the clearance process. The requirement of laforin for malin to degrade misfolded proteins resembles the findings on PTG protein, wherein the PTG was found to interact with laforin, and the presence of laforin was essential for the malin-mediated degradation of PTG (33,34). Thus, laforin appears to be a critical partner for malin's cellular functions.

The protein quality control system is essential for the removal of abnormal and long-lived proteins from the cellular milieu. This system involves two groups of proteins: one that includes molecular chaperones, such as heat shock proteins, that recognize and aid in converting misfolded proteins to functional forms (35). The other group represents factors involved in the UPS pathway that degrades severely damaged or misfolded proteins to release the amino acid residues (3). In recent years, there are growing numbers of reports that document a functional link between chaperone systems and proteolysis machinery, suggesting that the two systems work together to maintain permissive cellular concentrations of certain short-lived and abnormal proteins (36). Because substrate recognition in the UPS is known to be regulated by the E3 ubiquitin ligases and as the malin–laforin complex appears to identify a diverse set of misfolded proteins, we thought that the malin–laforin complex might use Hsp70 as an essential component in this functional unit. We show here that Hsp70 indeed interacts with the malin–laforin complex and its presence is critically essential for the complex to clear misfolded proteins. The specific affinity of Hsp70 to misfolded proteins has been well documented (37,38). Thus, Hsp70 might confer the apparent broader range of substrate specificity that the malin–laforin complex had displayed in the present study. Consistent with this view, malin was unable to interact with misfolded proteins when Hsp70 was knocked down. However, laforin was able to pull the misfolded proteins even in the absence of Hsp70, suggesting that laforin–misfolded protein interaction was independent of Hsp70. This interaction could possibly be mediated by chaperones other than Hsp70. In fact, chaperones such as Hsp27 and HDJ-2 are known to interact with and ameliorate the toxicity of misfolded proteins (7,10) and therefore it would be of interest to check the interactions between these chaperones and LD proteins. Intriguingly, we found that the overexpression of Hsp70 was unable to reduce the toxicity of misfolded proteins when laforin or malin was knocked down. Thus, Hsp70 appears to function as a complex that also includes malin and laforin and that the LD proteins are essential for the complex to act on misfolded proteins. To further support this model, we show here that the malin–laforin complex interacts with Hsp70 even in the absence of misfolded proteins, suggesting that the laforin and malin...
or overexpression of Hsp70 results in the reduction of either arresting the expression of mutant huntingtin protein proteins might regulate the aggregation property and the levels of the malin–laforin complex and the misfolded ongoing attempt by the LD protein complex in ‘clearing’ the toxic forms (42). The recruitment of laforin and malin to ally distinct intermediates that eventually lead to the insoluble protein with expanded glutamine repeats forms conformationally toxic of misfolded proteins by preventing and/or reducing malin–laforin complex perhaps rescues the cells from the toxicity of misfolded proteins.

The present report strengthens our suggestion and calls for further studies on the possible role of misfolded proteins in the etiology of LD. In conclusion, this study highlights the importance of the malin–laforin complex in promoting the clearance of misfolded proteins via proteasomal degradation. This study thus opens up a new and attractive therapeutic pathway for polyglutamine and other neurodegenerative disorders associated with toxic misfolded proteins.

MATERIALS AND METHODS

Reagents and antibodies

Chemicals MG132, tunicamycin, bafilomycin and 3MA were purchased from Sigma-Aldrich India Pvt Ltd. The following antibodies were used in the present study: anti-GFP (Roche, India), anti-Myc (Cell Signaling Technology, USA), anti-γ-tubulin (Sigma-Aldrich India Pvt Ltd), anti-Hsp70 (Sigma-Aldrich India Pvt Ltd), anti-FLAG (Sigma-Aldrich India Pvt Ltd), anti-ubiquitin (Dako, Denmark), anti-laforin (Abnowa, Taiwan, Republic of China) and anti-malin (Antibodies Incorporated, USA). Anti-huntingtin antibody (S830) was generously provided by Gill Bates. Secondary antibodies were purchased from Sigma-Aldrich India Pvt Ltd. The following chemicals were used in the present study: anti-GFP, anti-Myc (Cell Signaling Technology, USA), anti-γ-tubulin (Sigma-Aldrich India Pvt Ltd), anti-Hsp70 (Sigma-Aldrich India Pvt Ltd), anti-FLAG (Sigma-Aldrich India Pvt Ltd), anti-ubiquitin (Dako, Denmark), anti-laforin (Abnowa, Taiwan, Republic of China) and anti-malin (Antibodies Incorporated, USA). Anti-huntingtin antibody (S830) was generously provided by Gill Bates. Secondary antibodies were obtained from Jackson Immuno Research Inc. (USA).

Expression constructs

The expression vectors containing Myc- or GFP-tagged wild-type or mutant forms of malin and laforin were described previously (18). For generating construct to express A20, and D20 repeats as fusion product to the GFP, two complementary oligonucleotides having mixed codons for the amino repeats (42). The recruitment of laforin and malin to huntingtin aggregates in the cellular model and in the transgenic mice could possibly represent the unsuccessful or an ongoing attempt by the LD protein complex in ‘clearing’ the aggregates through the UPS. Thus, an imbalance between the levels of the malin–laforin complex and the misfolded proteins might regulate the aggregation property and the toxicity. Studies in mice models have indeed shown that either arresting the expression of mutant huntingtin protein or overexpression of Hsp70 results in the reduction of nuclear aggregates and the disease severity (6,43). Thus, the overexpression of the malin–laforin complex could enhance the functions of the cellular quality control system and rescue the cells from the toxicity of misfolded proteins.

The carbohydrate-rich Lafora polyglucosan bodies are hallmark of LD (44,45). However, no proteinaceous inclusions are known in LD; therefore, the relevance of the present findings to the LD pathology is unclear as of now. It should be noted however that Lafora bodies were stained positive for ubiquitin and advanced glycation end-products (AGEP), suggesting the presence of abnormal proteins in the polyglucosan aggregates (46). AGEPs are insoluble and non-degradable products that develop from the interaction between reducing sugars and long-lived proteins and are known to generate oxidative stress on proteins (46). As abnormal/damaged proteins are known to be removed by the proteasomal system (47,48), we have recently suggested that malin defects in LD may lead to increased abnormal protein load in the neurons (18).

We have shown here that the overexpression of LD proteins alters the aggregation property of expanded huntingtin. The malin–laforin complex perhaps rescues the cells from the toxicity of misfolded proteins by preventing and/or reducing the formation of aggregates that are conformationally toxic to the cell. It has indeed been shown recently that huntingtin protein with expanded glutamine repeats forms conformationally distinct intermediates that eventually lead to the insoluble toxic forms (42). The recruitment of laforin and malin to huntingtin aggregates in the cellular model and in the transgenic mice could possibly represent the unsuccessful or an ongoing attempt by the LD protein complex in ‘clearing’ the aggregates through the UPS. Thus, an imbalance between the levels of the malin–laforin complex and the misfolded proteins might regulate the aggregation property and the toxicity. Studies in mice models have indeed shown that either arresting the expression of mutant huntingtin protein or overexpression of Hsp70 results in the reduction of nuclear aggregates and the disease severity (6,43). Thus, the
were a gift from Peter Lansbury (Harvard Medical School, Boston, MA, USA). The pcDNA3 expression plasmids encoding wild-type HA-tagged Hsp70 were gift of Harm H. Kampinga (University Medical Center Groningen, The Netherlands). The antisense Hsp70 pcDNA3 plasmid, containing a 500 bp fragment of the human hsp70 cDNA in the antisense orientation (974–475 bp), was a kind gift of M. Jaattela (Institute for Cancer Biology, Denmark) (49). The mutant ubiquitin construct (K48R) was provided by Lih-Shen Chin (Emory University School of Medicine, USA), and the wild-type ubiquitin construct was generated by amplifying the coding sequence from cDNA and cloning into a pcDNA 3.1 vector. The pCMV-LacZ construct was generously provided by Jonaki Sen (Indian Institute of Technology, Kanpur, India). The RNAi constructs for laforin and malin (shRNAmir) were purchased from Open Biosystem, USA (Expression Arrest™ microRNA-adapted shRNA libraries) and were validated for their efficiency of knockdown (see Supplementary Material, Fig. S4).

Figure 9. The malin–laforin complex suppresses the cellular toxicity of misfolded proteins with the help of Hsp70. (A) Levels of toxicity of tHtt-Q97-GFP in COS-7 cells co-expressing malin and Hsp70 or its antisense construct (AsHsp70) (49). (B) Levels of toxicity of tHtt-Q97-GFP in COS-7 cells co-expressing laforin and Hsp70 or its antisense construct (AsHsp70). (C) Overexpression of Hsp70 does not suppress the toxicity of misfolded proteins in the absence of malin or laforin. COS-7 cells were co-transfected with the construct for Htt-Q97-GFP, overexpression or a knockdown construct for Hsp70 and knockdown construct for laforin or malin as indicated, and the toxicity was scored. The value of each bar represents the mean of three independent transfections, with a minimum of 300 cells scored for each set. Error bars represent the SD of the mean. The P-value < 0.005, calculated by a paired t-test, is denoted over the bar by double asterisks (**). The efficiency of knockdown by the AsHsp70 construct was established earlier (48) and in the present study by immunoblotting (see Supplementary Material, Fig. S4B). (D) COS-7 cells were transfected with the expression construct coding Myc/His-tagged malin, laforin or an empty vector (pcDNA) and processed for pull-down assay using the Ni-affinity resin. The pulldown products (PD) and the whole cell lysates (WCLs) were immunoblotted (IB) with anti-Hsp70 and anti-Myc antibodies, as indicated. (E) COS-7 cells were transiently co-transfected with a combination of various constructs as indicated in the figure and processed for pull-down assay using the Ni-affinity resin. The PD and the WCLs were IB with anti-Hsp70 and anti-Myc antibodies, as indicated. (F) COS-7 cells were transiently transfected with expression construct for GFP-D20, antisense Hsp70 (AsHsp70) and laforin or malin and processed for pull-down assay using the Ni-affinity resin. The PD and the WCLs were IB with anti-GFP and anti-Myc antibodies, as indicated.

Cell culture and transfection
COS-7 and Neuro2A cells were grown in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich India Pvt Ltd) supplemented with 10% (v/v) fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were grown at 37°C in 5% CO2. Transfection was performed using LipofectAMINE 2000 transfection reagent (Invitrogen Inc., USA), according to the manufacturer’s protocol. Cells were harvested at 24 h or later as indicated.

Blockers for autophagy–lysosome and proteasome pathways
Transiently transfected cells were allowed to express the desired proteins for 12 h and then incubated for further 12 h in the medium containing one of the following inhibitors; 20 μM MG132 (Calbiochem, USA), 25 mM ammonium chloride (Merck, India), 10 mM 3-methyladenine (3-MA, Sigma-Aldrich India Pvt Ltd) or 200 nM bafilomycin A1 (Sigma-Aldrich India Pvt Ltd). Control experiments using the resuspension solvents dimethyl sulfoxide or distilled water were performed where appropriate. See legend to Figure 8 for details on blockers.

Pulse-chase analysis
The COS-7 cells were transiently transfected with an inducible version of expression construct that codes for His-tagged
tHtt-Q97-GFP (28) and wild-type or the mutant forms of malin (C26S) or laforin (Q293L) (18). Twenty four hours post-transfection, the cells were treated with ponasterone A (5 \mu M/ml) for 12 h. The cells were then washed with phosphate-buffered saline (PBS) and harvested at 0, 2.5, 5 or 10 h with the addition of a fresh medium.

Immunocytochemistry and counting of aggregates and apoptotic cells

COS-7 cells, grown on gelatin-coated sterile glass cover slips, were fixed and processed for immunofluorescence microscopy essentially as described earlier (18). 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 \mu M 4',6-diamidino-2-phenylindole (DAPI). GFP-positive cells showing apoptotic bodies (see Supplementary Material, Fig. S1H and K) or aggregate formation (for tHtt-Q97-GFP) (Fig. 10A) were manually counted under the fluorescence microscope (250 transfected cells in each set), and the cells containing more than one aggregate were considered to have a single aggregate. Experiments were repeated at least thrice, and counts were made in a blinded manner.
Pull-down experiments
To establish the physical interaction between malin or laforin and other target proteins, we used the expression constructs that code for Myc/His-tagged malin and laforin (18) or the His-tagged tHtt-Q97-GFP (28). Lysates of cells that had expressed His-tagged malin or laforin with the desired protein were incubated with Ni-affinity resin (Sigma-Aldrich India Pvt Ltd) for 1 h at 4°C and processed for pull-down assays as recommended by the manufacturer. Pulled-down products were detected by immunoblotting using specific antibodies.

Ubiquitination assay
Cells that were expressing the His-tagged tHtt-Q97-GFP (either alone or along with laforin or malin) were processed for the Ni-affinity pull down assay, as described above. The pulled-down products were transferred to a membrane and reacted with specific antibodies.

Immunoblotting analysis
Protein samples were run on a 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose filter (MDI, India), as described previously (18). After blocking with 5% non-fat dry milk powder in 1 × PBS, the membranes were processed through sequential incubations with primary antibody, followed by secondary antibody at dilutions as recommended by the manufacturer. Immunoreactive proteins on the filter were visualized using a chemiluminescent detection kit (SuperSignal West PICO, Pierce, USA).

Statistical analysis
Standard deviations (SDs) for the observed values were calculated and plotted for every experiment, and statistical significance was tested with two-tailed paired t-test (P < 0.05) using the GraphPad software.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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formation in neuronal and non-neuronal cells overexpressing Parkin.


