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Structural basis for mutation-induced destabilization of profilin 1 in ALS

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*Mutations in profilin 1 (PFN1) are associated with amyotrophic lateral sclerosis (ALS); however, the pathological mechanism of PFN1 in this fatal disease is unknown. We demonstrate that ALS-linked mutations severely destabilize the native conformation of PFN1 in vitro and cause accelerated turnover of the PFN1 protein in cells. This mutation-induced destabilization can account for the high propensity of ALS-linked variants to aggregate and also provides rationale for their reported loss-of-function phenotypes in cell-based assays. The source of this destabilization is illuminated by the X-ray crystal structures of several PFN1 proteins, revealing an expanded cavity near the protein core of the destabilized M114T variant. In contrast, the E117G mutation only modestly perturbs the structure and stability of PFN1, an observation that reconciles the occurrence of this mutation in the control population. These findings suggest that a destabilized form of PFN1 underlies PFN1-mediated ALS pathogenesis.*

amytrophic lateral sclerosis | profilin 1 | protein stability | X-ray crystallography | protein misfolding

M
tuations in the profilin 1 gene (PFN1) were recently associated with both familial and sporadic forms of amyotrophic lateral sclerosis (ALS) (1, 2), an incurable and fatal neurodegenerative disease that primarily targets motor neurons (3). The etiology of sporadic ALS is poorly understood, whereas familial ALS is caused by inheritable genetic defects in defined genes such as PFN1 (3). PFN1 is a 15-kDa protein that is best known for its role in actin dynamics in the context of endocytosis, membrane trafficking, cell motility, and neuronal growth and differentiation (4). In addition to binding monomeric or G-actin, PFN1 also binds to a host of different proteins through their poly-proline motifs and to lipids such as phosphatidylinositol 4,5-bisphosphate (4, 5). However, little is known about the mechanism(s) associated with PFN1-mediated ALS pathogenesis. The observation that most ALS-linked PFN1 variants are highly prone to aggregation in mammalian cultured cells suggests that disease-causing mutations induce an altered, or misfolded, conformation within PFN1 (2). Protein misfolding is a hallmark feature of most neurodegenerative diseases, including ALS (3), and can contribute to disease through both gain-of-toxic-function and loss-of-normal-function mechanisms (6). Although mutations in PFN1 cause ALS through a dominant inheritance mode (2), there is some evidence supporting a loss-of-function mechanism for mutant PFN1. For example, ALS-linked mutations were shown to abrogate the binding of PFN1 to actin (2) and to impair the incorporation of PFN1 into cytoplasmic stress granules during arsenite-induced stress (7) in cultured cells. Moreover, ectopic expression of these variants in murine motor neurons led to a reduction in both axon outgrowth and growth cone size, consistent with a loss of function through a dominant-negative mechanism (2).

Although ALS-linked mutations were shown to induce PFN1 aggregation, the effect of these mutations on protein stability and structure has not been studied. Because the impact of disease-causing mutations on protein stability varies from protein to protein (8–10), these parameters must be determined empirically. Here, we demonstrate that certain familial ALS-linked mutations severely destabilize PFN1 in vitro and cause faster turnover of the protein in neuronal cells. To gain insight into the source of this mutation-induced instability, the 3D crystal structures for three PFN1 proteins, including the WT protein, were solved by X-ray crystallography. We discovered that the M114T mutation created a cleft that extended into the interior of PFN1. Further, we predict that the most severely destabilizing C71G mutation also creates a cavity near the core of the PFN1 protein, proximal to the cleft formed by M114T. Experimental mutations that create enlarged pockets or cavities are known to exert a destabilizing effect on the protein’s native conformation (11), and there are several examples of mutation-induced cavity formation occurring in nature and disease (12, 13). Interestingly, the variant predicted to be the least pathogenic according to recent genetics studies, E117G, was relatively stable and closely resembled the WT protein in every assessment performed herein (2, 14). These data implicate a destabilized form of PFN1 in ALS pathogenesis and call for therapeutic strategies that can stabilize mutant PFN1.

Results

ALS-Linked Mutations Destabilize PFN1 in Vitro. To investigate the effect of ALS-linked mutations on the stability of PFN1, PFN1 proteins were expressed and purified from Escherichia coli and subjected to chemical and thermal denaturation analyses. A novel purification protocol that includes sequential cation-exchange and gel filtration chromatography steps was developed.

Significance

Mutations in profilin 1 (PFN1) were recently shown to cause amyotrophic lateral sclerosis (ALS); however, little is known about the pathological mechanism of PFN1 in disease. We demonstrate that ALS-linked mutations cause PFN1 to become destabilized in vitro and in cells, likely through a mechanism that involves mutation-induced cavities within the protein core. Changes in protein stability due to disease-causing mutations can play a pivotal role across different disease mechanisms. The destabilized mutant-PFN1 species identified here can serve as an upstream trigger for either loss-of-function or gain-of-toxic-function mechanisms and thus emerges from these studies as a pertinent therapeutic target for the incurable disease ALS.


The authors declare no conflict of interest.

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Data deposition: The crystallography, atomic coordinates, and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 4X1L, 4X1M, and 4X25).

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Boopathy et al.

ALS-Linked Mutations Induce a Misfolded Conformation Within PFN1. We reasoned that ALS-linked variants must undergo some degree of structural or conformational change to account for their destabilization. However, ALS-causing mutations did not perturb...
the secondary structural elements of PFN1 as determined by CD spectroscopy (Fig. S4), and the fact that similar m values were determined for all PFN1 variants by the urea denaturation analysis suggested these proteins adopt similar tertiary structures as well (Table 1) (18). To probe further for potential structural differences between PFN1 WT and ALS-linked variants, these proteins were subjected to native gel electrophoresis, a biochemical technique capable of detecting conformational differences between misfolded variants and their WT counterparts (19). PFN1 WT and E117G migrated predominately as single, distinct bands with similar mobility, whereas multiple bands of slower mobility were observed for PFN1 variants C71G, M114T, and G118V (Fig. S5A). The slower mobility bands likely reflect the larger hydrodynamic volume due to partial unfolding of these variants. In addition, PFN1 C71G, M114T, and G118V produced relatively large-molecular-weight species that were retained in the stacking gel and unable to electrophorese through the separating native gel but were resolublized under conditions used for the denaturing gel (Fig. S5A). Analytical size-exclusion chromatography revealed that all PFN1 proteins eluted as expected for soluble, monomeric PFN1 (Fig. S5 B-G). However, despite equal loading of PFN1 proteins onto the analytical size-exclusion column, the peak area corresponding to soluble monomer PFN1 is reduced for ALS-linked variants, particularly for the most aggregation-prone variant, C71G. These data are consistent with a loss of soluble monomer PFN1 in the form of insoluble species that cannot pass through the analytical size-exclusion column filter.

**A Source of Mutation-Induced Destabilization Revealed by X-Ray Crystallography of PFN1.** Crystal structures of PFN1 proteins were determined to identify regions within mutant PFN1 that are conformationally distinct from PFN1 WT at atomic resolution. PFN1 WT, E117G, and M114T produced crystals that diffracted at relatively high resolution (∼2.2 Å; Table S1). The 3D structure of human PFN1 WT agrees well with previously determined structures (20–22). PFN1 WT and E117G crystallized in the same space group, C212, whereas M114T crystallized in the P6 space group, with two molecules (designated as chains A and B) in the asymmetric unit (Table S1).

Residues 22–36, 46–52, 101–105, 112–120, and 125–128 within PFN1 were used for Cα superimposition of the four molecules (PFN1 WT, M114T chains A and B, and E117G). In agreement with the biochemical analyses described above (Table I and Fig. S4), the secondary and tertiary structures of all three PFN1 proteins, including chains A and B of M114T, are highly similar (Fig. 3). Although the space groups for PFN1 WT and M114T crystals were different, we calculated the double difference plots between these and the other PFN1 structures to get a sense for structural perturbations potentially induced by the ALS-linked mutations. Double difference plots were constructed by calculating the distances between all of the Cα atoms in PFN1 WT and an ALS-linked variant separately, and then plotting the difference of the difference between PFN1 structures as described previously (23). Virtually no structural deviations were observed between PFN1 WT and E117G, whereas moderate differences were detected between WT and M114T (Fig. S6).

Next we sought to determine whether these moderate structural changes between PFN1 WT and M114T mapped to regions involved in PFN1 function, namely to residues that make contact with actin (24–31) or poly-γ-proline (21, 22, 24, 32, 33). The ternary complex comprised of PFN1 WT, actin, and the poly-γ-proline peptide derived from vasodilator-stimulated phosphoprotein (VASP) (21) (PDB ID code 2PAV) is shown in Fig. 4. Residues with the highest (0.3 Å or greater) average absolute double difference (Avg-Abs-DD) values between PFN1 WT and M114T chain B (Fig. S6C) were mapped onto PFN1 WT (Fig. S7). PFN1 M114T chain B was used for this and all subsequent structural comparisons because chain B had lower B factors compared with chain A (Fig. S8). Indeed, several PFN1 residues that reportedly make contacts with actin (V119, H120, G122, and K126) and poly-γ-proline (W4, Y7, H134, and S138) also have relatively high Avg-Abs-DD values (Fig. S7).

To assess whether these mutation-induced structural changes are sufficient to alter the normal binding interactions of PFN1,
we first monitored changes in the intrinsic tryptophan fluorescence of PFN1 as a function of poly-L-proline peptide concentration (Fig. S4). Our results revealed that the effect of ALS-linked mutations on the PFN1-poly-L-proline interaction was modest, because the apparent dissociation constants (K_d) were within twofold for all PFN1 proteins in this study (Table 1). In fact, excess concentrations of poly-L-proline effectively stabilized all PFN1 proteins as determined by DSF, with the largest increase in T_m observed at 100 μM poly-L-proline (Fig. S8 and Table 1). Next, we measured the binding capacity of our PFN1 proteins for G-actin by comparing their concentration-dependent abilities to suppress spontaneous polymerization of pyrenyllosoacetamide-labeled actin monomers (34). This assay is based on the fact that PFN1 binds G-actin and inhibits actin nucleation in the absence of formins (34).

As expected, increasing concentrations of recombinant PFN1 WT reduced the rate of actin polymerization, whereas the H120E variant that exhibits impaired binding to actin failed to suppress actin polymerization to the same extent (Fig. 6). Of the four ALS-linked variants, only G118V was defective in suppressing actin polymerization, which was most apparent at the highest concentration of PFN1 used in this assay, although this effect did not reach statistical significance (Fig. 6). These data argue against a general mechanism for PFN1-mediated ALS pathogenesis that involves impaired direct binding between PFN1 and either poly-L-proline or actin.

Importantly, the X-ray crystal structures reveal a possible mechanism by which ALS-linked mutations destabilize PFN1. Residues Thr90, Met114, and Gin18 contribute to the formation of a surface exposed pocket that was detected using SiteMap (Fig. 7). Mutation of methionine to threonine at position 114 increased the size of this pocket, thereby forming a cleft, because the residues nearby failed to rearrange and compensate for the loss of van der Waals contacts (Fig. 7B). This cleft is expected to exert a destabilizing effect on the native conformation of PFN1 owing to this loss of van der Waals contacts and the reduced hydrophobicity of the threonine side chain relative to that of methionine (11). Moreover, hydrophobic residues that are otherwise buried in the PFN1 WT structure were exposed by the cleft in the PFN1 M114T structure (Fig. 7 and Fig. S9). To investigate the potential impact of the C71G mutation on PFN1 structure, the cysteine side chain of residue 71 was removed to mimic a glycine amino acid in the PFN1 WT structure, and the PFN1 WT structure was visualized using PyMOL. Interestingly, this mutation is predicted to form a void in the core of the protein that partially overlaps with the cleft observed in the PFN1 M114T crystal structure (Fig. 7B). Analysis using PyMOL and SiteMap suggest that, unlike the solvent-accessible WT and the M114T pocket, the proposed C71G void is buried within the core of the protein. Solvent-inaccessible voids have a more destabilizing effect than solvent-exposed cavities (11, 35), providing an explanation for why the C71G mutation is more destabilizing than M114T (Fig. 1).

Discussion

Here we show that ALS-linked mutations severely destabilize (Fig. 1) and alter the native protein conformation (Fig. 3) of PFN1. Changes in protein stability owing to disease-causing mutations, whether these mutations stabilize or destabilize the protein, are thought to play a pivotal role in various disease mechanisms (13). In the context of ALS, disease-linked mutations destabilize Cu, Zn-superoxide dismutase (SOD1) (9), but instead hyperstabilize TAR DNA-binding protein 43 (TDP-43) (8, 10, 36). These findings underscore the importance of defining the toxic properties of disease-linked proteins, thereby directing the rational design of therapeutic strategies against those offending proteins (3). Our X-ray crystal structures of PFN1 proteins illuminate a probable source of mutation-induced destabilization. An enlarged surface pocket, or void, forms as a result of the M114T mutation (Fig. 7). The destabilizing effect of similar voids has been demonstrated using a systematic site-directed mutagenesis approach with lysozyme and is thought to arise from a loss of hydrophobic interactions (11, 35). Examples of mutation-induced cavity formation and destabilization have also been observed in nature (13). Interestingly, modeling the removal of the cysteine side chain at position 71 creates an internal cavity that is predicted to partially overlap the cleft formed by M114T, raising the intriguing possibility that both mutations destabilize PFN1 through a common mechanism that involves the loss of hydrophobic and van der Waals contacts within the same region of PFN1 (Fig. 7). Because
The misfolding of PFN1 variants may also induce gain of toxic functions and interactions, the latter via aberrant protein–protein interactions through exposed hydrophobic patches, such as those detected for PFN1 M114T (Fig. S9). Further, the aggregation of PFN1 variants can potentially sequester other vital proteins, including those with poly-L-proline binding motifs (4), culminating in compromised actin and/or cellular homeostasis (6).

Although the downstream effect of ALS-linked PFN1 on actin dynamics and other cellular processes have not been elucidated, our data identify misfolded and destabilized PFN1 as a potential upstream trigger of the adverse events that culminate in ALS, opening new avenues for therapeutic advancement in ALS. One potential direction is the development of pharmacological chaperones (16). For example, small molecules that fill the void formed by the M114T mutation are expected to stabilize the protein (35). Our data with poly-L-proline (Fig. S8) suggest that small-molecules binding to other regions of PFN1 could also stabilize the protein. We posit that stabilizing mutant PFN1 will restore the normal structure and function of the protein, thereby preventing the pathogenic cascade leading to ALS.

Materials and Methods

A pET vector containing human PFN1 flanked by Ndel and EcoRI restriction sites was kindly provided by Bruce Goode, Brandeis University, Waltham, MA. The mutant PFN1 DNA (2) was amplified using primers 5′-GGACCA-TATGCGCGGTGACCA-3′ and 5′-GCCGTAATCTCACTGCGAAGACC-3′ and ligated into the pET vector using Ndel and EcoRI restriction sites. BL21 (DE3) pLyS cells (20132; Aligent Technologies) transformed with PFN1 constructs were cultured in LB containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol at 37 °C until an OD600 of 0.7, at which point PFN1 expression was induced by addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG; Amresco) for either 3 h at 37 °C (for WT and E117G) or 24 h at 18 °C (for C71G, M114T, and G118V). Cells were harvested by centrifugation and stored until purification. Refer to Supporting Information for complete details on methods.

Fig. 6. The binding of PFN1 proteins to G-actin. Polymerization of monomeric rabbit muscle actin (3 μM, 5% pyrene-labeled) was monitored in the presence of increasing concentrations of WT or ALS-linked PFN1 variants and used to derive relative rates of polymerization (n = 3). The variant H120E, which is impaired in binding to actin, fails to suppress spontaneous actin polymerization as effectively as WT PFN1. Although G118V is relatively weak in suppressing actin polymerization, the data did not reach statistical significance. Statistical significance was determined using a two-way ANOVA followed by a Tukey’s post hoc analysis. **p < 0.01 for WT vs. H120E at 7 μM concentration. No other significant comparisons with WT were obtained. Other significant comparisons included C71G vs. H120E and E117G vs. H120E (p ≤ 0.05) at 7 μM concentration. Error bars represent SD.

Fig. 7. The M114T mutation causes a surface-exposed pocket to expand into the core of the PFN1 protein. (A) Residues are depicted as described in Fig. 3. The van der Waals radii of residues 90, 114, and 18 are in contact in the PFN1 WT structure (Top). These contacts are reduced by the M114T mutation (Bottom) owing to the smaller size of threonine, leading to an enlargement of the surface-exposed pocket. (B) PFN1 WT is shown with a transparent surface and the secondary structure is shown in cartoon representation. The surface pocket volume for PFN1 WT (green) and the cleft volume for PFN1 M114T chain B (red) are depicted as opaque surfaces and were generated using SiteMap. The predicted cavity (blue) for PFN1 C71G (generated using PyMOL) overlays with the M114T void, and unlike the WT and M114T volumes, is not surface-exposed. The insets (Right) show the aforementioned voids for WT (Top), M114T chain B (Middle), and C71G (Bottom).
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