Manipulating the aggregation activity of human prion-like proteins

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ABSTRACT. Considerable advances in understanding the protein features favoring prion formation in yeast have facilitated the development of effective yeast prion prediction algorithms. Here we discuss a recent study in which we systematically explored the utility of the yeast prion prediction algorithm PAPA for designing mutations to modulate the aggregation activity of the human prion-like protein hnRNPA2B1. Mutations in hnRNPA2B1 cause multisystem proteinopathy in humans, and accelerate aggregation of the protein in vitro. Additionally, mutant hnRNPA2B1 forms cytoplasmic inclusions when expressed in Drosophila, and the mutant prion-like domain can substitute for a portion of a yeast prion domain in supporting prion activity in yeast. PAPA was quite successful at predicting the effects of PrLD mutations on prion activity in yeast and on in vitro aggregation propensity. Additionally, PAPA successfully predicted the effects of most, but not all, mutations in the PrLD of the hnRNPA2B1 protein when expressed in Drosophila. These results suggest that PAPA is quite effective at predicting the effects of mutations on intrinsic aggregation propensity, but that intracellular factors can influence aggregation and prion-like activity in vivo. A more complete understanding of these intracellular factors may inform the next generation of prion prediction algorithms.

KEYWORDS. amyloid, prion, protein aggregation, prion-like domains, yeast

Numerous prions in yeast result from the structural conversion of proteins into an insoluble amyloid form (for review, see1). Most of the yeast prion proteins contain a glutamine/asparagine (Q/N) rich prion domain that drives prion formation. Hundreds of human proteins contain prion-like domains, defined as regions with high amino acid compositional similarity to the yeast prion domains.2 Recently, mutations in several of these PrLD-containing proteins have been linked to various degenerative disorders, including amyotrophic lateral sclerosis (ALS), frontotemporal dementia, and inclusion body myopathy (for review, see2,3) Therefore, there has been considerable interest in understanding how mutations in prion and prion-like proteins affect aggregation propensity.

Prion formation by Q/N-rich yeast prion proteins appears to be driven primarily by amino acid composition, not primary sequence.4,5 We previously developed a quantitative mutagenesis method to empirically estimate the prion
propensities of the 20 canonical amino acids within the context of a Q/N-rich prion domain. Amino acids with similar physicochemical properties tend to have similar prion propensity scores, suggesting that basic physicochemical properties of the amino acid groups are largely responsible for their effects on prion formation. These prion propensity scores allowed us to develop a prion prediction algorithm (which we named the Prion Aggregation Prediction Algorithm, or PAPA). PAPA is reasonably effective at predicting the prion-like activity of Q/N-rich yeast PrLDs and at predicting the effects of mutations on yeast prion domains.

In a recent study, we examined whether PAPA would be similarly proficient at predicting the effects of mutations in human prion-like proteins. The human heterogeneous nuclear ribonucleoprotein hnRNPA2B1, which is expressed as 2 isoforms (A2 and B1), provides a useful system to examine this question. An aspartic acid to valine substitution in the prion-like domain of hnRNPA2B1 causes mislocalization and aggregation of the protein, resulting in multisystem proteinopathy in humans. When expressed in Drosophila, the wild-type protein is predominantly detergent-soluble and localized to the nucleus, while the mutant protein is largely detergent-insoluble, forms cytoplasmic inclusions, and causes muscle degeneration. In vitro, the hnRNPA2 protein (as well as the related hnRNPA1 protein) can undergo liquid-liquid phase separation, while the disease-associated mutations in both proteins accelerate conversion to an insoluble amyloid form. Finally, in yeast, the core PrLD from mutant hnRNPA2 can support prion activity when substituted into the prion nucleation domain of the yeast prion protein Sup35. Thus, hnRNPA2 offers a variety of experimental systems to examine the effects of mutation on aggregation activity.

RATIONALLY MANIPULATING THE AGGREGATION ACTIVITY OF A HUMAN PRION-LIKE DOMAIN

PAPA accurately predicts that the disease-associated D290V mutation in hnRNPA2 should increase aggregation propensity. While this is encouraging, a single mutant is clearly too small a sample size to draw any broad conclusions. According to our prion propensity estimates, aspartic acid is not expected to be uniquely prion inhibiting, and valine is not expected to be uniquely prion-promoting. Additionally, since prion formation is predominantly composition-dependent, the precise location of a given mutation within a PrLD should not dramatically alter its effects. To test these predictions, and to more comprehensively investigate PAPA’s prediction accuracy, we designed a range of single and double mutations categorized as prion-promoting, neutral, or prion-inhibiting based on PAPA’s predictions.

Because of the ease of assaying prion activity in yeast, we initially tested a set of 25 mutants in yeast. As demonstrated previously, when the wild-type hnRNPA2 PrLD was inserted in the place of the portion of Sup35 responsible for prion nucleation (amino acids 1–40), the resulting fusion displayed little to no prion activity; by contrast, the disease-associated D290V mutation dramatically enhanced its prion formation in yeast. We tested 8 additional substitutions at the 290 position, and found in all cases that PAPA accurately predicted their effects on prion formation. Furthermore, PAPA successfully predicted the combinatorial effects of various mutations at alternative positions. This demonstrates that PAPA is a good predictor of the prion propensity of human PrLDs when expressed in yeast, even when the mutations differ from those observed to cause disease in humans.

Additionally, PAPA accurately predicted the effects on aggregation and associated cellular pathology of most hnRNPA2 mutations in Drosophila. However, in a few cases, mutations resulted in unexpected deviation from PAPA predictions, and from our results in yeast. For example, PAPA predicts that tyrosine and isoleucine will have similar effects on prion formation. As predicted, the “neutral” Y283I substitution (in combination with the D290V mutation) maintained high rates of prion formation in yeast. However, the Y283I mutation completely restored the solubility of hnRNPA2(D290V) in Drosophila.
We used *in vitro* experiments to examine the mutants that showed divergent behavior between yeast and *Drosophila*. A protein’s aggregation *in vivo* will depend on both its intrinsic aggregation propensity and its interactions with other cellular factors. In each case where the *Drosophila* and yeast results diverged, the *in vitro* aggregation kinetics were consistent with the yeast results (and with PAPA predictions). This suggests that both PAPA and our yeast system accurately predict intrinsic aggregation propensity, but that additional cellular factors may influence aggregation in *Drosophila*.

It should be noted that this ability to design mutations to modulate intrinsic aggregation propensity may not be unique to PAPA. A subsequent reanalysis of our data set showed that PrionW is also reasonably effective at predicting prion activity in yeast for our mutants, and that combining PrionW and PAPA may further improve prediction accuracy.\(^{15}\) PrionW differs from PAPA in that it combines amino acid composition analysis with a position-specific matrix to identify predicted amyloid-prone segments.\(^{16}\) Other algorithms show varying degrees of predictive success. ZipperDB uses structure-based modeling to identify 6-amino-acid peptides with a high predicted propensity to form steric zipper segments.\(^{17}\) For all aggregation-promoting mutations at the disease-associated position in hnRNPA2, ZipperDB predicted that the mutations would create a strong steric zipper.\(^{10,11}\) However, when we designed subsequent mutations to specifically test the importance of these predicted steric zippers, we found that the presence of predicted steric zippers was neither necessary nor sufficient for strong aggregation activity in yeast or *in vitro*.\(^{10}\) ArchCandy predicts the probability that a sequence will adopt a \(\beta\)-arch.\(^{18}\) Prion-promoting mutations tended to increase the number of predicted \(\beta\)-arches, or the scores for the predicted \(\beta\)-arches, although in some cases these effects are only seen by adjusting the default scoring threshold (data not shown). Other algorithms were less effective. For example, PLAAC\(^{19}\) correctly identifies the PrLD in hnRNPA2,\(^{11}\) but was not effective at predicting the effects of mutations;\(^ {10,11}\) Aggrescan\(^ {20}\) fails to identify aggregation hot spots in most of the aggregation-prone mutants (data not shown); and TANGO\(^ {21}\) shows a relatively poor correlation between predicted and observed aggregation activity (data not shown).

**FUTURE CHALLENGES IN THE PREDICTION OF PRION-LIKE AGGREGATION**

The ability to rationally design mutations to modulate aggregation activity will provide a powerful tool for examining functional and pathogenic aggregation by PrLDS. An emerging theory suggests that many PrLDS have evolved to form dynamic, reversible interactions that mediate the formation of functional assemblies, including stress granules and P-bodies, and that mutations may cause disease by disrupting the dynamics of these assemblies.\(^ {3}\) Consistent with this theory, the cytoplasmic inclusions observed for disease-associated hnRNPA1 and A2 mutants are RNA-protein granules.\(^ {11,22}\) However, the limited number of naturally occurring pathogenic mutations makes this theory difficult to test. Additionally, most mutations experimentally designed to test the role of PrLDS in functional or pathogenic aggregation have involved substantial changes in protein sequence, such as deletion or substitution of the entire PrLD (for example, see\(^ {23,24}\)). Such mutations may have other effects on the protein. By contrast, we observed dramatic changes in aggregation with just single and double point mutations.\(^ {10}\) Thus, it should be possible to design a variety of mutations in other PrLDS to more rigorously explore the role of PrLDS in functional and pathogenic aggregation.

However, our results also demonstrate a fundamental challenge in predicting protein aggregation: although methods like PAPA can be quite effective at predicting the intrinsic aggregation propensity of proteins (Pathway 1 in Fig. 1), in a complex cellular environment, a variety of interactions may influence aggregation (Fig. 1). These other factors are not currently incorporated into any prion prediction algorithm. It is worth noting that some
prediction algorithms were derived from analysis of sequences that demonstrate bona fide prion activity in yeast. Therefore, to some degree, they indirectly integrate the cumulative influence of an intracellular environment into their predictions; however, this may also limit their adaptability to other organisms.

Indeed, while it is encouraging that most mutants showed similar behavior in yeast, Drosophila and in vitro, the rare deviations among these systems highlight a limitation of using any single model system to study protein aggregation. Without understanding the basis for these differences, it is unclear whether the yeast or Drosophila results will be more predictive of human disease.

Here we discuss some of the factors that may influence in vivo protein aggregation, many of which were evident among our panel of mutants. While it may not be feasible to take into account all of these factors on a protein-by-protein basis, a better understanding of how these factors influence aggregation activity could lead to better-informed predictions.

Protein Expression and Abundance

Aggregation is a concentration-dependent process, so intracellular protein concentrations could affect the probability of self-association and prion formation. By extension, the free intracellular concentration of a given protein might be affected by the concentrations and affinities of molecular interacting partners, such as RNA or other proteins. For example, β-thalassemia results from mutations that decrease β-globin levels, resulting in an imbalance in the ratio of α-globin to β-globin, and aggregation of the excess α-globin. Thus, the aggregation is not caused by a change in α-globin’s aggregation propensity, but instead by a change in the concentration of its binding partner. Protein levels and molecular interacting partners vary from organism to organism, from tissue to tissue, and even between subcellular compartments, potentially explaining why many aggregation-based diseases only affect specific tissues. Mutations could therefore affect aggregation by altering protein expression or stability, or by disrupting stabilizing protein-protein interactions.

Distinct Steps in Protein Aggregation

The formation of amyloid aggregates involves at least 2 steps: nucleation and fiber growth. Additionally, because amyloid fibers grow from their ends, fragmentation of fibers allows for the exponential amplification of amyloid aggregates by creating new ends for fiber growth. Each of these steps likely has distinct sequence/compositional requirements, yet existing prediction algorithms do not separately examine the effects of mutations on each step. Furthermore, because each of these steps is likely influenced by distinct sets of chaperone proteins (for review, see ), the different chaperone environments in different cells types or organisms may further affect the sequence requirements for prion-like aggregation.

One of our double-mutants, D276V/D290V, appeared to show unique aggregate dynamics in Drosophila. As predicted, these prion-promoting mutations had an additive effect on the rate of prion formation in yeast, and resulted in the formation of cytoplasmic aggregates in Drosophila. However, the aggregates in Drosophila were greater in number and smaller in size compared with those formed by the D290V single mutant, suggesting that the dynamics of aggregate formation or fragmentation had been fundamentally altered.

Liquid-Liquid Phase Separation

A number of recent studies demonstrate that many proteins with PrLDs undergo concentration-dependent liquid-liquid phase separation (Pathway 2 in Fig. 1) under a variety of conditions, including changes in salt concentration, RNA concentration, poly(ADP)-ribose, pH, and temperature (for review, see ). In many cases, the PrLDs play a direct role in liquid-liquid de-mixing, or affect de-mixing indirectly. This biological process has been proposed as the basis for the formation of various non-membrane-bound organelles, and can occur in both the cytoplasm (e.g., stress granules and P-bodies).
and the nucleus (e.g., nucleolus, nucleolar sub-compartments, Cajal bodies, nuclear paraspeckles, etc.). Protein de-mixing greatly enhances the local concentrations of incorporated proteins, which may increase the likelihood of forming stable amyloid-like aggregates. Mutations within PrLDs may also perturb the dynamics of these assemblies, which can result in the formation of off-pathway pathological aggregates (Pathway 3 in Fig. 1). It is also unclear whether differences in subcellular environments (particularly, between the nucleus and the cytoplasm) can affect PrLDs differently based on their sequence and composition. Further investigation is necessary to develop a more complete mechanistic understanding of the
biophysical conditions and protein features that govern liquid-liquid phase separation, which may ultimately inform prion prediction methods.

**Aggregation, Proteostasis, and Aging**

For most proteins, misfolding and aggregation represent a cytotoxic threat to cells. Accordingly, cells possess an arsenal of protein quality control factors to maintain proper proteostasis. The ability to evade the proteostasis machinery may be an important protein trait when considering aggregation propensity in vivo. Furthermore, cellular proteostasis typically declines with age (for review, see 33), so aggregation-promoting mutations in PrLDs coupled with an age-related decline in cellular proteostasis may contribute to the late-onset nature of many neurodegenerative diseases. Mutations could affect aggregation by altering interactions with the proteostasis machinery, which includes protein chaperones and protein degradation systems (Pathways 4 and 5 in Fig. 1). A deeper understanding of the rules governing cellular recognition and handling systems of aggregation-prone proteins may facilitate improvements in prion prediction methods.

**Nucleocytoplasmic Shuttling of Prion-Like Proteins**

Many of the disease-linked PrLD-containing proteins are predominantly nuclear, but form cytoplasmic inclusions in diseased patients. This has led some to propose that disruption of nucleocytoplasmic transport is a key mediator of disease pathology (for review, see 34). Aberrant transport dynamics of PrLD-containing proteins could lead to accumulation of the protein in the cytosol, which may increase aggregation risk (Pathway 6 in Fig. 1). A particularly well-characterized example is the PrLD-containing protein, fused in sarcoma (FUS); many ALS-associated mutations in FUS directly impair its nuclear import and lead to accumulation in cytoplasmic aggregates. 35 Although it is clear that nucleocytoplasmic transport is affected in disease, it is currently unclear whether subcellular localization and trafficking dynamics directly influence or simply coincide with protein aggregation. Understanding how mutations alter intracellular trafficking dynamics may shed light on why many of these proteins form cytoplasmic inclusions.

Intriguingly, for one of our mutants, aggregation and cytoplasmic mislocalization were uncoupled. Consistent with PAPA predictions, a D290F mutation enhanced prion formation in yeast and formation of detergent insoluble aggregates in *Drosophila*. However, while hnRNPA2(D290V) formed cytoplasmic inclusions, hnRNPA2(D290F) aggregates appeared to localize to the nucleus, suggesting that while both mutations increase aggregation propensity, the mutations have different effects on nucleocytoplasmic transport dynamics. Such mutants may also provide insight into the relative roles of aggregation and mislocalization in cellular pathology.

**Post-Translational Regulation of Prion-Like Proteins**

Post-translational modifications within or near PrLDs could affect intrinsic aggregation propensity, liquid-liquid phase separation, intracellular trafficking, targeted degradation, or a variety of other processes. The human PrLDs tend to contain a high proportion of glutamine, asparagine, serine, and tyrosine residues (for review, see 36), which can all be modified post-translationally. Additionally, the hnRNPA1 and hnRNPA2 PrLDs (as well as other PrLD-containing proteins, including FUS, EWSR1, and TAF15; for review, see 37) possess a small number of arginine residues within or near the PrLDs that are asymmetrically di-methylated. 38,39 Methylation of these residues in the hnRNPs or similar PrLD-containing proteins has been shown to regulate nucleocytoplasmic shuttling, liquid-liquid phase separation, liquid-liquid phase separation, stress granule assembly, and pathological protein aggregation. 35,40

Therefore, post-translational modifications can affect directly or indirectly a multitude of intracellular processes that relate to protein aggregation. Mutations may affect aggregation
by altering these post-translational modifications. Indeed, in *Drosophila* the insoluble fraction of hnRNPA2(D290V) appears as 2 distinct bands by western blot, suggesting a post-translational modification. This double-band was not observed for any of the other hnRNPA2 mutants (including the D276V/D290V double mutant), and this modification is not detectable when the D290V PrLD was expressed in yeast. It is unclear whether this modification affects aggregation or pathology.

**CONCLUSION**

Our intention is to highlight the interplay between intrinsic prion domain features and prion formation in a cellular context. A generalized understanding of the features governing these prion-modifying pathways may offer ways to explicitly weight prion predictions with additional intracellular considerations. This will be especially important when extending prion predictions to multicellular eukaryotic organisms, in which organism-specific and tissue-specific intracellular conditions may differ substantially.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

No potential conflicts of interest were disclosed.

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