Protein misfolding in Dictyostelium: Using a freak of nature to gain insight into a universal problem

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ABSTRACT. Prion-like proteins can undergo conformational rearrangements from an intrinsically disordered to a highly ordered amyloid state. This ability to change conformation is encoded in distinctive domains, termed prion domains (PrDs). Previous work suggests that PrDs change conformation to affect protein function and create phenotypic diversity. More recent work shows that PrDs can also undergo many weak interactions when disordered, allowing them to organize the intracellular space into dynamic compartments. However, mutations within PrDs and altered aggregation properties have also been linked to age-related diseases in humans. Thus, the physiological role of prion-like proteins, the mechanisms regulating their conformational promiscuity and the links to disease are still unclear. Here, we summarize recent work with prion-like proteins in *Dictyostelium discoideum*. This work was motivated by the finding that *D. discoideum* has the highest content of prion-like proteins of all organisms investigated to date. Surprisingly, we find that endogenous and exogenous prion-like proteins remain soluble in *D. discoideum* and do not misfold and aggregate. We provide evidence that this is due to specific adaptations in the protein quality control machinery, which may allow *D. discoideum* to tolerate its highly aggregation-prone proteome. We predict that *D. discoideum* will be an important model to study the function of prion-like proteins and their mechanistic links to disease.

CHOOSING THE RIGHT MODEL

The ability of prion-like proteins to misfold poses a challenge to cellular and organismal health. It is therefore of utmost importance to understand the mechanisms that control their conformational promiscuity.

The majority of studies on misfolding-prone prion-like proteins have been done with classical model organisms, such as yeast, worms or flies. These studies have provided valuable insights. However, the narrow focus on a few models may have biased our perception of prion-like proteins.

Model organisms are tremendously powerful drivers of biomedical research, and the choice of a model organism often plays an important role in the success of a research project. Experimental accessibility has long been the key criterion for choosing a model organism. However, with increasing advances in technology, such as CRISPR/Cas9-mediated genome engineering, this feature is becoming less important. Instead, the challenge today is to find a model with specific biological features. These features may have been amplified or exaggerated by evolution, and could thus provide insight into an important aspect of biology. Prominent examples are organisms with an extraordinary capacity to self-renew, such as planarian flatworms or axolotl.[1] Studies carried out in these organisms allowed important insight into molecular mechanisms of regeneration. Thus, a model organism with amplified features can provide a unique window into biology, often with very broad implications.

DICTYOSTELIUM: A MODEL FOR PROTEIN MISFOLDING AND AGGREGATION

Protein aggregates are a hallmark of many age-related neurodegenerative diseases. The aggregated proteins share a common feature: they posses regions that are devoid of protein structure, so-called intrinsically disordered regions. The compositional complexity of these sequences is low and they often contain a high content of polar, uncharged amino acids, such as glutamine (Q) and asparagine (N). Sequences with such amino acid compositions show similarity to regions in yeast prion proteins and are thus often referred to as prion-like domains. Yeast prion proteins can switch from a non-prion to an aggregated prion state, and this switch is associated with a change in protein function.[2,3] Once formed, these prion states are highly stable and can spread as epigenetic elements in a population of yeast cells. They have been proposed to be benign drivers of phenotypic diversity in clonal populations of single-celled organisms such as budding yeast.[4] However, in humans, proteins with prion-like domains have been implicated in neurodegenerative diseases,[5,6] and mutations in PrDs often accelerate disease progression.[7-9]

Research on yeast prion proteins has provided important insight into the structural basis and the functional role of amyloids and prions.[10,11] In an effort to gain further insight into this class of proteins, we recently expanded the repertoire of model organisms for the study of prion-like proteins. This was driven by the observation that proteomes, or, more specifically, the amino acid composition of proteomes, are vastly different from organism to organism. Previous comparative studies revealed that the highest content of Q/N-rich low complexity sequences is found in the social amoebae *Dictyostelium discoideum*.[12] Thus, we hypothesized that this natural enrichment of Q/N-rich domains might have enabled the evolution of sophisticated mechanisms to control and maintain them.

To investigate this idea, we first reevaluated the Q/N richness of the *D. discoideum* proteome. In agreement with previous results, we observed that the number of proteins containing homopolymeric runs of Qs or Ns exceeds by far the numbers observed in other organisms such as *S. cerevisiae* or humans.[13] Moreover, the polyQ or polyN runs often extend beyond the aggregation threshold of 35–40 residues (see **Table 1**). In addition, we identified 1,733 proteins in the proteome of *D. discoideum* with prion-like sequence features. These findings indicate that the proteome of *D. discoideum* might have a much higher aggregation propensity compared to other organisms. Given this observation, we hypothesized that *D. discoideum* could provide a unique window into the universal problem of protein misfolding and aggregation.

PRION-LIKE DOMAINS ARE CONSERVED AND MAY PLAY AN IMPORTANT ROLE DURING DEVELOPMENT

The accumulation of prion-like domains within the proteome of *D. discoideum* seems puzzling, because the high aggregation

TABLE 1. Proteins with homopolymeric N or Q stretches above the aggregation threshold of 35 residues

Species	polyN > 35	polyQ > 35
D. discoideum	632	118
S. cerevisiae	–	1
H. sapiens	–	12

propensity of these domains can be an extraordinary liability to a cell. Therefore, we hypothesized that there may be a strong selective pressure against the continued presence of PrDs. Interestingly, however, we found ~900 prion-like proteins in the proteome of the close relative *D. purpureum*.[13] Of these, 505 proteins are shared between both species. We also found a comparable overlap within the associated protein super-domain families. This contradicts the notion of a random distribution of prion-like domains and argues that the domains have functions that are under positive selection. This notion is further substantiated by the observation that the identified prion-like domains in *D. discoideum* are significantly associated with RNA binding domains (RRM), an observation that was also made in *S. cerevisiae*, humans and other organisms.[14,15] Furthermore, a fraction of the identified prion-like proteins cluster into specific gene ontology (GO) groups in *D. discoideum* (**Fig. 1**) as well as in other organisms.[13] Interestingly, the identified groups in all organisms represent similar cellular components and molecular functions. These findings point toward a shared biological function of prion-like domains in different organisms.

The content of intrinsically disordered proteins within a proteome differs between species. The amount of disorder seems to be related to the habitat of a species, with increasing levels of disordered proteins found in organisms, which live in very unstable environments.[16] *D. discoideum* is a soil living amoebae and therefore faces constant environmental fluctuations. Moreover, it has a fascinating live cycle, where, upon nutrient depletion, the organism changes from a unicellular state to a multi-cellular state (**Fig. 2A**). During this transition, differentiation takes place, and cells are primed to either differentiate into surviving spores or into dying stalk cells. Surprisingly, we found a significant functional enrichment of prion-like proteins in developmental processes (6.7% in prion-like proteins compared to 3,7% in the whole proteome, p < 0.005) (**Fig. 2B**). This indicates that prion-like proteins may be involved in regulating the developmental cycle of *D. discoideum*. This hypothesis agrees with previous suggestions, which link an increase in morphological complexity with increased levels of intrinsic disorder in a proteome.[16] However, the question remains how prion-like proteins function on the molecular level. Recent findings by the Verstreppen lab suggest that repeat variations in Q/N-rich domains can tune the solubility of proteins and thus their functional state.[17] Because the frequency of mutations in repeat regions is much higher than anywhere else in a protein sequence, one possible function of these repeats could be that they accelerate adaptive mutations in highly fluctuating environments.

D. DISCOIDEUM *EFFICIENTLY CONTROLS THE AGGREGATION OF PRION-LIKE PROTEINS*

The accumulation of aggregation-prone prion-like proteins in the proteome of *D. discoideum* raises the question whether this organism has evolved mechanisms to control the aggregation of these proteins. To approach this question, we studied the aggregation behavior of several well-characterized prion-like proteins. These proteins have been studied extensively in other organisms such as yeast, *C. elegans*, or mammalian cells and found to form cytosolic aggregates.[18-20]

First, we studied proteins containing homopolymeric polyQ or polyN runs. We used a polyQ-containing version of the human Huntingtin exon 1 with 103 glutamines (Q103) and a synthetic variant in which the glutamines were replaced by 47 asparagines (N47). Both proteins exhibit similar aggregation properties in *S. cerevisiae*.[21] In contrast to observations made in other organisms, when expressed in *D. discoideum* cells, these proteins remained

FIGURE 1. Prion-like proteins cluster into specific gene ontology groups. Within the group of prion-like domains, specific gene ontology (GO) groups are enriched. (**A**) Proteins associated with the cytoskeleton and the nucleus are specifically enriched among prion-like proteins. 622 proteins of the identified 1733 correspond to these groups. (**B**) Prion-like proteins are enriched for GO terms associated with DNA binding, RNA binding, DNA modification, protein modification and signaling. 661 proteins of the identified 1733 correspond to these groups.
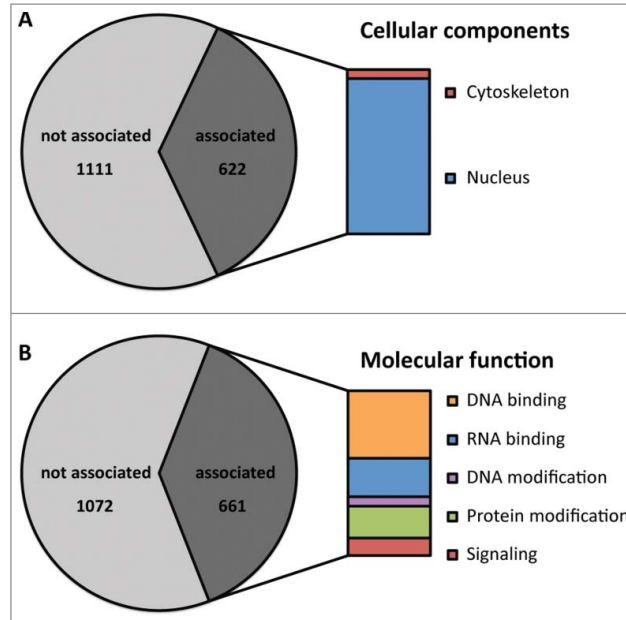

diffusely distributed in almost all cells.[13] However, we detected small amounts of aggregated material in biochemical assays, which suggests that the proteins may aggregate in a small fraction of the cells. We think that these few cells are stressed, mostly likely though damage or aging. The amount of aggregated material was slightly higher in cells expressing N47 than in cells expressing Q103, suggesting that N47 is more aggregation-prone than Q103. We attribute this to the different effects of asparagine and glutamine on amyloid formation, as reported previously.[21,22] Interestingly, in a small number of N47-expressing cells, N47 also accumulated in the nucleus.[13] Thus, we conclude that N47 can accumulate in the nucleus, but that N47 and Q103 remain mostly soluble in *D. discoideum.*

Next, we analyzed the aggregation behavior of prion-like proteins, first focusing on the PrD (NM) of the yeast prion proteins Sup35. Consistent with observations made for Q103 and N47, we observed a diffuse distribution of NM throughout the cytosol and barely any accumulation of aggregated material.[13] When we expressed a modified version of NM, which was engineered to have a higher aggregation propensity in yeast, we observed a slight increase in the aggregated material and a higher incidence of nuclear accumulations. In addition, we analyzed the behavior of endogenous prion-like domains. These proteins remained likewise soluble in *D. discoideum*, while they formed cytosolic aggregates when expressed in *S. cerevisiae*.[13] These observations indicate that proteins, which have a high tendency to aggregate in other organisms, remain soluble when expressed in *D. discoideum*. This supports the hypothesis that *D. discoideum* possesses sophisticated pathways to control the aggregation of prion-like proteins.

We next performed a detailed analysis of the nuclear signal. We noticed that 75% of the cells showed a diffuse nuclear distribution of prion-

FIGURE 2. Prion-like proteins may be involved in the development of *D. discoideum*. (**A**) Live cycle of *D. discoideum*. Upon nutrient depletion, cells enter the developmental cycle and proceed through several morphogenic states during which the cells differentiate into spore cells or stalk cells. Spore cells remain dormant until the conditions become more favorable. (**B**) Prion-like proteins are specifically enriched for proteins associated with developmental processes. 155 proteins are involved in general or specific processes during sorocarp development.
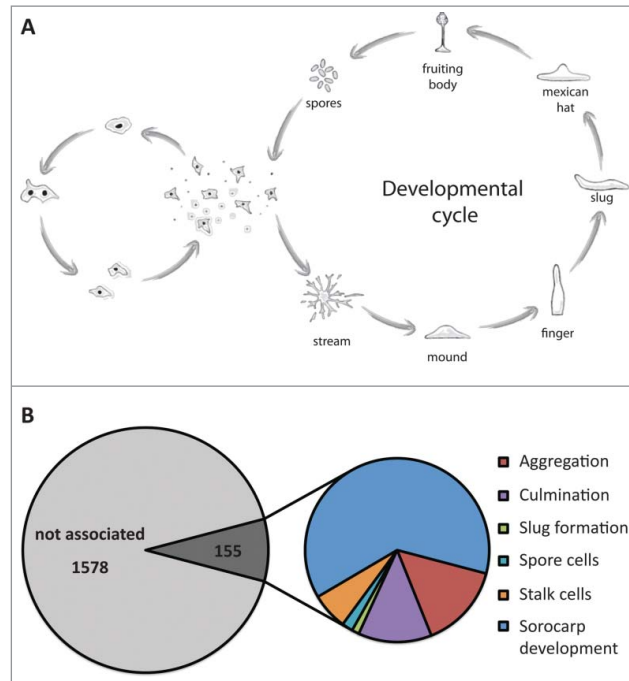
like proteins with occasional small puncta. In the remaining 25%, the proteins localized to 2 or 3 distinct nuclear foci.[13] Subsequent analysis by correlative light and electron microscopy (CLEM) and immunostainings of histones and DNA suggest that these structures represent nucleoli. To test whether the nuclear accumulations contain aggregated proteins, we measured their mobility using fluorescence recovery after photobleaching (FRAP). Surprisingly, the nuclear foci showed a high mobility, thus excluding the possibility that the nuclear accumulations consist of amyloid. Rather, this suggests that prion-like proteins are enriched in nuclear compartments like the nucleolus.

Why do prion-like proteins accumulate in the nucleus of *D. discoideum*? Several previous studies showed that cytosolic aggregation-prone proteins accumulate in the nucleus and are subsequently degraded.[23,24] The nuclear localization of N47 and modified NM indicates that similar processes might occur in *D. discoideum*. Indeed, we found that proteins accumulating in the nucleus are ubiquitinated and their degradation is reduced when proteasomal inhibitors are added.[13] These observations substantiate the notion that the nucleus of *D. discoideum* serves as a compartment for protein quality control, wherein aggregation-prone proteins accumulate and are degraded by the ubiquitin proteasome system (UPS). Interestingly, previous studies showed that, in other organisms, the UPS is impaired through accumulating polyQ proteins, most likely because they are difficult to degrade.[25] This suggests that *D. discoideum* may have undergone specific adaptations, which decrease the vulnerability of the ubiquitin proteasome system to these proteins.

AGGREGATION OF PRION-LIKE PROTEINS IS CONTROLLED BY MOLECULAR CHAPERONES

The surprising observation that prion-like proteins, both exogenous and endogenous, remain soluble in *D. discoideum* suggests that cytosolic pathways control protein aggregation. Indeed, we found that impairment of the molecular chaperone system, either by specific chaperone inhibitors or heat stress, causes massive formation of cytosolic aggregates in *D. discoideum*.[13] Moreover, we observed that the formed cytosolic aggregates could be dissolved once the stress was removed. We attribute this function to the disaggregase Hsp101, a chaperone of the Hsp100 family and ortholog of the yeast Hsp104. Overexpression of the disaggregase prevented heat-induced formation of aggregates, and inhibition reduced the dissolution of aggregates in the recovery phase.[13] This suggests that *D. discoideum* has evolved mechanisms to prevent aggregation under normal growth conditions and to reverse aggregation after stress. During both molecular processes chaperones play a key role. Taken together, these findings show that *D. discoideum* has evolved several molecular mechanisms to control aggregation-prone proteins. During normal growth, proteins can be efficiently degraded by the proteasome and can be kept soluble by molecular chaperones (**Fig. 3**). In a second layer of defense, *D. discoideum* utilizes the disaggregase Hsp101 and probably also other chaperones to ensure survival during stress conditions (**Fig. 3**).

In addition to the mechanisms described above, cells can also use mitosis to generate aggregate-free progeny. This has been shown for asymmetrically dividing cells such as *S. cerevisiae*,[26] but also in higher eukaryotes.[27] Although *D. discoideum* divides predominantly symmetrically, it undergoes asymmetric cell sorting during development. Thus, by sorting damage-containing cells to prestalk regions during development (**Fig. 2A, B**). discoideum may be able to clean the population of unfit cells. The mechanisms underlying the fate decision in *D. discoideum* have been controversially discussed for many years. Interestingly, however, stressful conditions increase the fraction of cells that commit to the stalk cell fate.[28] This suggests that *D. discoideum* might use its developmental program to purge the population of cells that have accumulated protein damage. Investigations are ongoing to study whether cell-sorting mechanisms have a role in purging *D. discoideum* of protein aggregates.

FIGURE 3. Molecular mechanisms controlling protein aggregation in *D. discoideum. D. discoideum* cells have evolved at least 2 mechanisms to control protein aggregation. During normal growth conditions, proteins are kept soluble by molecular chaperones and can be degraded in the nucleus. The disaggregase Hsp101, probably in combination with other chaperones, can rescue stress-induced protein aggregation and restore cellular integrity.
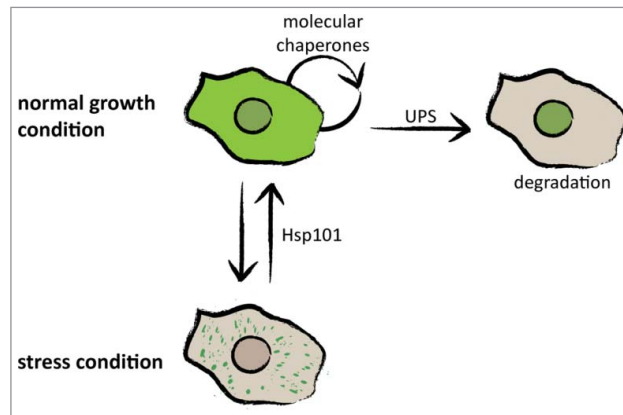
CAN D. DISCOIDEUM *HELP US DETERMINE THE FUNCTIONAL ROLE OF PRION-LIKE DOMAINS?*

Although we have gained important insight into the mechanisms regulating the conformational promiscuity of prion-like proteins, we still have a very limited understanding of their function in living cells. It is unlikely that all prion-like proteins in a given proteome form amyloid-like assemblies. Such an overwhelming presence of amyloid structures would be difficult to control. Instead, a newly emerging concept suggests that prion-like domains may primarily act through transiently forming protein-protein contacts, which aid in the formation of dynamic and disordered assemblies in living cells.[9,29] According to this view, the functional state of many prion-like domains is not the amyloid-like state but the disordered state. Thus, we think that *D. discoideum* will not only help us understand the mechanisms controlling prion-like proteins, but it will also aid in solving the controversy of the functional role of prion-like domains.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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