What makes a good prion?

Conference on Prion Biology

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The Cold Spring Harbor Laboratory/Wellcome Trust Conference on Prion Biology took place between 7 and 11 September 2005 in Hinxton, UK, and was organized by A. Aguzzi, B. Chesebro, M. Tuite and R. Wickner.

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alike. Consequently, one of the interesting features of the prion field is that it brings together people from different backgrounds. The joint Cold Spring Harbor Laboratory/Wellcome Trust Prion Biology meeting was probably the first international prion meeting intended as a cross-talk platform between mammalian and fungal prionologists. In addition to the mammalian disease-causing prions, reports dealt with the yeast [*PSI*⁺], [*URE3*] and [*PIN*⁺] prions, the [*Het-s*] prion of the filamentous fungus *Podospora anserina* and new fungal prions.

Many fundamental questions in prion research remain unanswered (Aguzzi & Polymenidou, 2004). What is the nature and structure of the infectious agent? How do prions replicate? How do they cause disease? Work presented at the meeting from both fungal and mammalian studies provided participants with new perspectives on these questions. For example, several complementary presentations provided strong support for a crucial role for amyloid in several models of prion propagation (Weissmann, 2005). Another principal theme that emerged during the meeting was an expansion of prion biology to include new questions about the normal biological functions of prions in cells (Shorter & Lindquist, 2005). Even if the haze of mystery surrounding prions is only gradually clearing, the participants felt that much progress has been made recently in this field.

The chemical and physical nature of prions

For decades, the most contentious issue in prion research has been the 'protein-only' hypothesis, which proposes that infectious prions are composed exclusively of misfolded proteins. Recent work has confirmed this unorthodox hypothesis for the [*PSI*⁺] and [*Het-s*] fungal prions, and several scientists addressed the chemical nature of the infectious agent in other prion systems. [*URE3*] is the prion form of a yeast protein called Ure2. In the first presentation of the meeting, R. Wickner (Bethesda, MD, USA) showed that the introduction of recombinant Ure2 amyloid fibres into yeast cells produces the [*URE3*] prion-infected phenotype. Interestingly, infectious material was always >20 nm in diameter, suggesting that the mechanism of propagation is a nucleation-polymerization process.

C. Soto (Galveston, TX, USA) described landmark experiments in which an automated form of the Protein Misfolding Cyclic Amplification (PMCA) technique was used to propagate wild-type

Introduction

Although transmissible spongiform encephalopathies have been studied for decades, prion biology is still a young discipline. The prion concept has existed for less than a quarter of a century, and the study of fungal prions is barely in its teens. The fascinating idea that a protein can turn into a deadly self-replicating entity attracts neurobiologists, cell biologists, geneticists and structural biologists

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infectious mammalian prions *in vitro* for the first time. Brain homogenates from normal and prion-infected animals were incubated together and intermittently sonicated. The sonication process probably breaks up prion aggregates into smaller 'seeds', which can then form more prions in an exponential manner. Animal bioassays confirmed that brain homogenates serially propagate PrP^{Sc} (the protease-resistant conformer of the mammalian prion protein) and prion infectivity *in vitro*. Later in the meeting, S. Supattapone (Hanover, NH, USA) presented additional data on the *in vitro* formation of mammalian prions. Supattapone and his colleagues used PMCA to generate PrP^{Sc} *in vitro* using purified PrP^C (the normal conformer of PrP) as a substrate. Ongoing bioassay experiments with these *in vitro*-generated, purified PrP^{Sc} molecules might eventually confirm that PrP^{Sc} molecules are the infectious agent of mammalian prion disease.

A longstanding goal in the field has been to determine the minimum size of the infectious prion particle. This information might help to resolve whether the mechanism of propagation involves a nucleation-polymerization or template-assistance process. Previously published radiation inactivation experiments suggest that the essential infectious particle is ~50–150 kDa, which corresponds to a PrP^{Sc} trimer. B. Caughey (Hamilton, MT, USA) reported his recent finding that the most infectious particles are ~300–600 kDa. Caughey's group used flow field-flow fractionation chromatography and light-scattering spectroscopy to separate and analyse prions that had been disrupted by sonication in sodium undecyl sulphate. After separation, they found no infectivity in fractions containing fewer than six PrP molecules. They also found that small aggregates are more infectious per mass unit than large aggregates. Specific infectivity decreases with increasing size. P. Lansbury Jr proposed as early as 1993 that prion propagation might be based on the nucleation-polymerization mechanism that underlies amyloid formation. Caughey's results can be interpreted in the context of the nucleation-polymerization model as increased specific infectivity in smaller PrP assemblies might reflect the higher number of fibril ends per mass unit. Several lines of evidence suggest that, in amyloid diseases, oligomers are more toxic to cells than large aggregates. This work shows that in the case of prion diseases, smaller aggregates are more infectious and thus potentially more pathogenic.

Strains, barriers, infectious and non-infectious amyloids

Many prionologists now believe that amyloid is the infectious agent in prion diseases. The present challenge is to gain insight into the structure of the amyloid state and to explain some of the biological features of prions. One counter-argument often used against the protein-only hypothesis is the existence of prion strains. In mammals, prion strains are defined by precise incubation periods and neuropathologic profiles. This information is somehow enciphered in the infectious particle and is faithfully transmitted and replicated. For the protein-only hypothesis, this requires the creation of several distinct propagating conformations from a single primary sequence. Until now, most prionologists thought this theory was too much at odds with Anfinsen's principle, which states that the amino-acid sequence of a protein contains all the information required for the formation of its final, native structure. However, recent reports have indicated that amyloid aggregates can be polymorphic; that is, a given protein can adopt different amyloid conformations and propagate them (Chien *et al*, 2004).

Several presentations provided compelling evidence to support this concept. J. Weissman (San Francisco, CA, USA) first described work showing that differences in amyloid structure underlie strain differences in the [PSI⁺] yeast prion, and discussed how these structural variations might translate to phenotypic differences. [PSI⁺] strains can be distinguished by using an ad hoc colour marker (Fig 1). Weissman showed that different prion strains can be generated *in vitro* by controlling the conditions in which the amyloid is formed (in this case the temperature). In atomic force microscopy (AFM) studies, he found that these amyloid strains differ in their polymerization rate and fragility, as measured by their susceptibility to mechanical shearing (Fig 1). This remarkable work poses a general and central question: what is the difference between a prion and a non-infectious amyloid? Weissman proposed a model in which the infectious nature of an amyloid is defined by two fundamental parameters: the rate of polymer elongation and its susceptibility to shearing. An amyloid aggregate can be propagated as a prion in a population of dividing cells only if—in combination—these parameters surpass a threshold. The strain phenotype is then determined by the precise values of these parameters, which are dictated by the intrinsic physical properties of the particular amyloid conformation or strain. This work and several other presentations emphasized the importance of aggregate shearing in prion maintenance, a connection that M. Ter-Avanesyan was the first to recognize.

R. Tycko (Bethesda, MD, USA) showed that the ability to propagate structural polymorphisms is not limited to prion proteins. He showed that Alzheimer's A β (1–40) peptide can adopt different amyloid conformations depending on experimental conditions (quiescent or agitated incubation). This structural polymorphism can be shown by electron microscopy and solid-state nuclear magnetic resonance (NMR). Remarkably, these structural differences are 'heritable' *in vitro* when soluble peptide is seeded with preformed fibrils. This infiltration of a prion concept in the Alzheimer's disease field led Tycko to suggest that, despite the fact that Alzheimer's is not transmissible, different strains of the disease might exist. Thus, depending on the initial misfolding event, several distinct amyloid conformations might be deposited in the brain and these could be associated with different rates and topological distribution of neuronal damage. R. Riek (La Jolla, CA, USA) proposed a new high-resolution structural model for A β (1–42), which he generated using a combination of high-resolution hydrogen exchange and site-directed mutagenesis. This new model is characterized by the existence of an intermolecular salt bridge, and it explains several previous experimental observations—such as the activity of peptide inhibitors and unidirectional fibre growth. It is possible that slight differences between the models proposed by Riek and Tycko can be explained by the existence of amyloid polymorphism and by the fact that these studies used different A β peptides.

W. Surewicz (Cleveland, OH, USA) also addressed the issue of fibril structure diversity using a truncated version of PrP (PrP23–144) as a model. He showed that PrP23–144 from different species (human, mouse and hamster) form fibrils with conformational differences that can be detected by Fourier transform infrared spectroscopy and AFM. For instance, mouse fibrils are segmented whereas hamster fibrils are smooth. Interestingly, hamster fibrils can seed mouse PrP23–144, but mouse fibrils cannot seed hamster PrP23–144. However, the mouse fibrils formed as a result of seeding by hamster amyloids are smooth and able to seed

hamster fibrils. Surewicz concluded that breaching of the species barrier depends on the ability of host PrP^C to adopt the conformation of the donor PrP^{Sc} seed (a hypothesis initially proposed by J. Collinge). Work on the [PSI⁺] yeast prion from the Weissman group led to similar conclusions. The emerging theory that species barriers can be overcome by certain conformational variants suggests that a species barrier can never be considered absolute.

Several structural examples for the amyloid form of prions were presented. Wickner showed that prion domains from Ure2 and Sup35 are 'shuffleable', meaning that a random sequence of the same amino-acid composition can function perfectly as a prion domain. Wickner suggested that these prion domains might adopt an in-register parallel β -sheet conformation that would allow homotypic interaction independent of the primary sequence. S. Lindquist (Boston, MA, USA) described how chemically modified single cysteine variants of the Sup35 prion-forming domain produced important topological information about fibril organization, in particular the unexpected 'head-to-head, tail-to-tail' interaction of the monomers within the fibril. This approach also allowed her to delimit some of the regions that show structural differences in different [PSI⁺] strains. Riek, C. Ritter (La Jolla, CA, USA) and A. Siemer (Zurich, Switzerland) presented a structural model for the prion domain of the [Het-s] prion of *Podospora*. They constructed this model using a combination of hydrogen exchange, solid-state NMR and site-directed mutagenesis. The well-defined resonances displayed by the solid-state NMR spectra of HET-s(218–289) indicate a high molecular order, close to that found in micro-crystalline samples. This degree of order seems unusual for amyloids, as Tycko illustrated through the comparison of HET-s(218–289) and Ure2(10–39) solid-state spectra.

Biological functions of prion proteins

A central concept addressed by many participants at the meeting was that prions could have both beneficial and harmful effects on cells. In particular, there was some debate as to whether yeast prions might have a beneficial role, or whether they only represent 'diseases' of yeast. Lindquist argued that the ability of yeast to form [PSI⁺] confers an evolutionary advantage, not only because the prion domain of Sup35 (the protein that causes [PSI⁺]) has been conserved in yeast for more than 800 million years, but also because the [PSI⁺] prion state is associated with a growth advantage under certain environmental conditions. She proposed that [PSI⁺] allows yeast to uncover new phenotypes and to fix these as new genetic traits. By contrast, Wickner argued that, out of 70 strains examined, no natural yeast isolates were infected with either [URE3] or [PSI⁺], and concluded that it is improbable that these prion states are beneficial to yeast. S. Saupe (Bordeaux, France) described how, in *Podospora anserina*, mediation of the normal process of heterokaryon incompatibility depends on the prion state of the HET-s protein. Therefore, [Het-s] is an example of a prion state that is necessary for normal biological function. It is interesting to speculate that the well-resolved solid-state NMR line spectra of HET-s fibres, described by Siemer, might reflect the evolution of a functional amyloid structure, in contrast to disease-associated amyloids.

Although the prion conformer of mammalian PrP is clearly associated with disease, it seems that the normal conformer, PrP^C, might protect neurons from cell death. A. LeBlanc (Montreal, Quebec, Canada) showed that PrP^C expression prevents Bax-mediated apoptosis in cultured human neurons. Bax is a main pro-apoptotic

Fig 1 | [PSI⁺] prion strain phenotypes are determined by properties of conformational variants of Sup35 amyloids. (A) [PSI⁺] prion stains that can be distinguished by colour using a reporter system are based on the propagation of distinct amyloid conformation of the Sup35 protein. These conformational variants, which can be generated *in vitro* by varying the temperature of fibril formation, differ in their functional properties such as the polymerization rates that can be measured using an atomic force microscopy (AFM)-based assay (B) that measures individual fibre growth rate. (C) An initial seed (not bound by the antibody) is detected as a thin fibre and the newly synthesized extension (bound by the antibody) appears as a thicker fibre. Note that in this example, fibril growth is asymmetric. Amyloid conformation variants also display different susceptibility to mechanical shearing (not shown). These two properties (fibril growth rate and shearing susceptibility) determine the strain phenotype and the ability of the amyloid conformation to propagate as a prion. Figure provided by J. Weissman.

protein of neurons and PrP inhibits the Bax conformational change that occurs as a first step in Bax activation. Therefore, prion protein acts as a true Bax inhibitor. Surprisingly, ectopic expression of PrP^C in the cytosol also prevented Bax-mediated apoptosis, and the cytoprotective action of PrP^C did not require direct interaction with Bax. Explanation of the specific signalling pathway by which PrP^C molecules normally prevent neuronal cell death remains an important task in the field.

Are PrP homologues also present in non-mammalian vertebrate species? Apparently, yes. Birds, frogs and turtles contain PrP molecules. E. Málaga-Trillo (Konstanz, Germany) showed that fish species express two genetic orthologues of mammalian PrP. Interestingly, despite their considerable sequence divergence, the globular domain of both fish and mammalian PrP^C molecules seem to be structurally and functionally conserved, suggesting that this region of vertebrate PrP^C molecules might harbour an essential biological activity. Importantly, reduced expression of one of the zebrafish PrP genes has produced the first known PrP loss-of-function developmental phenotype, suggesting a conserved biological function of PrP during vertebrate development.

Special issues with mammalian prions

Although fungal systems have proven to be useful models for studying fundamental questions about prion biology and structure, some specific questions about the pathogenesis of clinical prion disease still need to be investigated in mammalian model systems. For instance, one question unique to multicellular animals concerns the mechanism by which infectious prions spread from cell to cell in the brain. Both R. Morris (London, UK) and Caughey reported significant progress in developing mammalian cell-culture systems to measure the uptake of sonicated, fluorescently labelled PrP^{Sc} fibres. Morris also described preliminary data suggesting that the LDL receptor protein (LRP) might be involved in the endocytosis of PrP^{Sc} molecules, and Caughey showed that the uptake of PrP^{Sc} molecules did not require the expression of PrP^C. Further work in this area should eventually identify a new therapeutic target based on preventing the cellular uptake of infectious prions.

B. Chesebro (Hamilton, MT, USA) presented remarkable results relevant to the pathogenesis of neurodegeneration in mammalian prion diseases. Chesebro's group genetically modified PrP to remove its glycosylphosphatidylinositol (GPI) anchor, expressed the secreted product in transgenic mice and infected these mice with scrapie prions. Surprisingly, the infected mice replicated infectious prions, but did not develop spongiform neurodegeneration. A specific histological stain revealed large deposits of amyloid, suggesting that this accumulation of amyloid aggregates does not cause neuronal cell death. An important question that remains to be answered is whether the mechanism of neurodegeneration in prion-infected, wild-type animals is cell-autonomous or whether it requires non-neuronal cells such as astrocytes or microglia.

It has long been observed that scrapie infection of sheep and chronic wasting disease (CWD) infection of deer can be transmitted horizontally, but the mode of horizontal transmission among herbivores remains unclear. A. Aguzzi (Zurich, Switzerland) presented exciting results showing that chronic inflammation of peripheral organs stimulates prion replication within the affected organs in mice (Fig 2). Aguzzi showed that an experimental form of lymphocytic nephritis causes concomitant prion replication in the affected kidney, accompanied by excretion of infectious prions in urine. These findings suggest that treating chronic organ inflammation in farm animals could be a simple strategy for preventing the lateral spread of prion diseases, and therefore might have significant implications for public health.

The birth of prions in yeast

S. Liebman (Chicago, IL, USA) described the existence of a prion network in yeast. This concept originates from her discovery of the $[PIN^-]$ prion—the prion version of an N/Q-rich protein called Rnq1 required for the *de novo* induction of $[PSI^-]$. Work on $[PIN^-]$ led to the remarkable conclusion that aggregates from other N/Q-rich proteins can facilitate the formation of Sup35 prions. $[PIN^-]$ can also influence the appearance of heterologous N/Q-rich or even non-N/Q-rich prions in yeast (Fig 3). This description of the *in vivo* interaction of unrelated amyloid prion proteins can have fundamental implications for other systems. The heterologous cross-seeding model, which shows that prion interactions require limited—if any—similarity at the primary sequence level, echoes Surewicz's finding on the role of conformational determinants in cross-species transmission. Heterologous cross-seeding can be viewed as an extreme case of species barrier breaching. As all amyloids share

Fig 2 | Histoblot analysis of prion-infected kidneys. Consecutive sections display colocalization of PrP^{Sc} deposits with follicular infiltrates of inflammatory cells (Hematoxylin & Eosin stain). Figure provided by A. Aguzzi.

Fig 3 | Heterologous prion cross-seeding model in yeast. $[PIN^-]$ prion aggregates can promote the appearance of $[PSI^-]$ prions by seeding aggregation of the Sup35 protein. This heterologous cross-seeding leads to formation of various $[PSI^-]$ strains. Original figure provided by S. Liebman.

common structural features, it is not surprising that highly divergent sequences can prime each other's polymerization. Prion–prion interactions seem to fall along a continuum stretching from efficient homotypic seeding to $[PIN^-]$ -type heterologous seeding, with cross-species transmission somewhere in between.

M. Tuite (Canterbury, UK) addressed the issue of spontaneous prion formation by using the $[PSI^-]$ system. He determined the frequency of spontaneous appearance of $[PSI^-]$ and investigated different hypotheses that could account for *de novo* $[PSI^-]$ spontaneous prion formation. He was able to prove that spontaneous $[PSI^-]$ do not occur as a result of somatic Sup35 gene mutations and showed that increasing the rate of translational errors did not increase the rate of spontaneous $[PSI^-]$ formation. The spontaneous emergence of $[PSI^-]$ thus remains a mystery. He also tried—but failed—to isolate $[PSI^-]$ in a $[pin^-]$ strain unless that strain had already become $[PIN^-]$, an observation also reported previously by Liebman's group. The discovery of the $[PIN^-]$ effect leads to a fundamental question: is there such a thing as *bona fide* spontaneous emergence of $[PSI^-]$? It is possible that a cellular structure—a constitutive prion, or maybe a soluble protein or protein complex—serves as a primer for $[PIN^-]$ generation and

Fig 4 | Yeast-based assay for the isolation of anti-prion drugs. **(A)** The molecule 6-aminophenanthridine was isolated in a yeast-based screening assay to isolate anti-prion drugs. **(B)** The assay is based on elimination of yeast prions detected as a white-to-red colour change when the drug is spotted on filter paper and placed on a lawn of prion-infected cells. **(C)** The molecule also leads to clearance of PrP^{Sc} in chronically infected mammalian cell lines. Elimination of PrP^{Sc} is detected using a western blot on proteinase K-treated samples. Figure provided by M. Blondel.

eventually leads to the emergence of [PSI⁺]. In other words, maybe there is always a pre-existing template for prion formation.

Conclusions

In summary, the meeting allowed participants to learn about the remarkable recent progress that has been made towards understanding the fundamental mechanism of prion propagation in fungal systems, as well as exciting new results relevant to mammalian prion disease. The timely interaction between prion researchers working in both fungal and mammalian systems produced several interesting scientific discussions and ideas. The potential benefit of integrated research using different systems was epitomized by M. Blondel's (Roscoff, France) presentation. Blondel and his colleagues have devised a method to identify new anti-prion drugs using a yeast-based screen. Using a high-throughput procedure, they identified four classes of compound that cured the [PSI⁺] and [URE3] prion phenotype in yeast (Fig 4). Remarkably, three of these drug classes were also potent inhibitors of prion replication in scrapie-infected mammalian cells and one drug compound corresponds to a molecule already in use in clinic for an unrelated pathology. Blondel's work reveals the existence of a prion-controlling mechanism common to yeast and mammals. The isolated compounds now represent valuable tools to dissect these shared mechanisms.

As a fitting conclusion to the meeting, C. Dobson (Cambridge, UK) delivered a broad analysis of amyloid formation, in which he discussed several of the main themes that had emerged during the meeting. Dobson explained that many proteins have the ability to form amyloids under the right folding conditions because amyloid structures are stabilized by main-chain interactions of the polypeptide backbone. Amyloids could be considered a 'primordial' structure, from which globular protein structure might have subsequently evolved (Dobson, 2004). Conversely, side-chain interactions govern the propensity for individual proteins to form amyloids, and it is possible that prion states caused by proteins prone to amyloid formation might represent 'post-evolutionary' diseases. Prion researchers working on fungal and mammalian systems can all look forward to future developments that seem certain to emerge from this dynamic and exciting field of research.

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