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'Prion-proof' for [*PIN*$^+$]: Infection with *in vitro*-made amyloid aggregates of Rnq1p-(132–405) induces [*PIN*$^+$]

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Summary

Prions are self-propagating infectious protein conformations. The mammalian prion, PrP^{Sc}, responsible for neurodegenerative diseases like mad-cow and Creutzfeldt Jacob's diseases, appears to be a β-sheet rich amyloid conformation of PrP^c that converts PrP^c into PrP^{Sc}. However, an unequivocal demonstration of 'protein-only' infection by PrP^{Sc} is still lacking. So far, "protein only" infection has been proven for three prions, [*PSI*$^+$], [URE3] and [Het-s], all of fungal origin. Considerable evidence also supports the hypothesis that another protein, the yeast Rnq1p, can form a prion, [*PIN*$^+$]. While Rnq1p does not lose any known function upon prionization, [*PIN*$^+$] has interesting positive phenotypes: facilitating the appearance and destabilization of other prions as well as the aggregation of poly-Gln extensions of the Huntingtin protein. Here, we polymerize a Gln/Asn-rich recombinant fragment of Rnq1p into β-sheet-rich amyloid-like aggregates. While the method used for [*PSI*$^+$] and [URE3] infectivity assays did not yield 'protein-only' infection for the Rnq1p aggregates, we did successfully obtain 'protein-only' infection by modifying the protocol. The work proves that [*PIN*$^+$] is a prion mediated by amyloid-like aggregates of Rnq1p, and supports the hypothesis that heterologous prions affect each others appearance and propagation through interaction of their amyloid-like regions.

Keywords

Amyloid-like; [*PIN*$^+$]; Prion; Protein-Only; Rnq1p

Introduction

The term 'Prion' was originally coined by Prusiner to refer to a mysterious infectious agent responsible for spongiform encephalopathies in cattle, scrapie in sheep and Kuru and Creutzfeldt-Jacob's disease in humans.[1,2] Remarkably, this infectious agent was resistant to hostile conditions such as UV, which would eliminate viruses. Such stability is expected only if the infectious entity is independent of any nucleic acid component. Therefore a 'protein-only' hypothesis was proposed which advocates that a prion is composed solely of a protein that can cause infection.[1,3] An unequivocal demonstration of 'protein-only' infection by a prion requires that the encoding protein, preferably expressed in a foreign host, be converted *in vitro* into the altered 'prion-conformation' and when introduced into a 'prion-free' cell be capable of transforming it into a 'prion-plus' cell.[4]

So far, only five well-known prions exist: PrP^{Sc} in mammals, [*PSI*$^+$], [*URE3*] and [*PIN*$^+$] in the yeast *Saccharomyces cerevisiae* and [Het-s] in *Podospora anserina*, respectively encoded

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by the proteins PrP^{C}, Sup35p, Ure2p, Rnq1p and HET-s.[2,5–8] The propagation of the fungal prions is strikingly similar to the mammalian prion, PrP^{Sc}. In all, the 'prion-conformation' of the encoding protein cooperatively sequesters the non-prionized protein into prion-aggregates, thereby depleting the protein's soluble pool.

Recent studies on the fungal prions, [Het-s],[9] [PSI_{+}][10,11] and [*URE3*][12] have shown that they propagate by a 'protein-only' mechanism. For these prions, the *in vitro*-made β-sheet-rich amyloid-like fibers of the encoding protein, that bear several conformational similarities[13–15] to the structures associated with the protein deposits in neurodegenerative disorders such as Huntington's and Alzheimer's diseases,[16,17] have been shown to be sufficient for infection of a [*prion*$^{-}$] cell to become [*PRION*$^{+}$]. The only prions for which 'protein-only' infection remained to be unambiguously demonstrated are PrP^{Sc} and [*PIN*$^{+}$]. Recent efforts to obtain the 'protein-only' infection by PrP^{Sc}, have come close to realizing this goal.[18–20]

The thus far least well-studied prion, [*PIN*$^{+}$], which gets its name for enhancing **P**si **In**duction, was identified as a non-chromosomal genetic element[21] and started a new concept of *in vivo* 'prion-prion' interactions[7] The propagation of [*PIN*$^{+}$] exhibits several similarities with the other yeast prions however, unlike [*PSI*$^{+}$] and [URE3], conversion to the [*PIN*$^{+}$] state is not associated with any known loss-of-function of the encoding Rnq1p, as its cellular function is not defined. On the contrary, in a [*PIN*$^{+}$] state, Rnq1p gains the function of facilitating the induction of another prion [*PSI*$^{+}$][7] as well as facilitating the aggregation of poly-glutamine extensions from the Huntingtin protein.[22]

Over-expression of Sup35p, the [*PSI*$^{+}$] prion protein, induces the *de novo* appearance of [*PSI*$^{+}$][5] and the efficiency of the *de novo* induction of [*PSI*$^{+}$] is greatly enhanced in the presence of [*PIN*$^{+}$].[7,21] Current evidence supports the hypothesis that Rnq1p aggregates in [*PIN*$^{+}$] yeast provide a nidus to initiate the *de novo* prionization of [*PSI*$^{+}$],[23] although it is also possible that [*PIN*$^{+}$] aggregates titrate out inhibitors of [*PSI*$^{+}$] prion formation.[7]

In addition to different 'strains' of the mammalian prion, which are distinguished with respect to disease symptoms, incubation time upon infection and brain regions affected in genetically identical hosts,[24–25] the prions in yeast also exhibit 'variants' which differ with respect to the intensities of the altered phenotype.[26–30] [*PIN*$^{+}$] can exist in different heritable variant phenotypes (e.g. low, medium, high and very high [*PIN*$^{+}$]) that differ in the efficiency with which they promote [*PSI*$^{+}$] induction[30] and also by fluorescent decoration patterns upon over-expression of the Rnq1-GFP fusion protein, with the high [*PIN*$^{+}$] showing prevalently multiple fluorescent dots whereas, the other three variants exhibit mostly a single dot per cell.[30]

Sup35p consists of three distinct regions: a Gln/Asn-rich N domain (aa: 1–123) which is essential for [*PSI*$^{+}$] induction and propagation,[26,31] a highly charged M domain (aa: 124–253) which facilitates differentiation of [*PSI*$^{+}$] variants[32,33] and a C domain (aa: 254–685) which is essential for the translation termination (eRF3) activity of Sup35p.[34] When the N domain of Sup35p was replaced with a Gln/Asn-rich fragment (aa: 153–405) of Rnq1p, the resulting chimeric protein propagated *in vivo* in a prion-like fashion (termed [*RPS*$^{+}$]).[6] Like [*PSI*$^{+}$] cells, [*RPS*$^{+}$] cells are impaired in translational termination activity due to sequestration of the translational release domain of Sup35p into prion aggregates. Thus, both [*PSI*$^{+}$] and [*RPS*$^{+}$] cause read-through of the premature nonsense mutation in *ade1-14* and therefore restore growth on medium lacking adenine (SC-Ade) and suppress the red color associated with the absence of Ade1p.

The findings of SDS-stable high molecular weight aggregates of Rnq1p in [*PIN*$^{+}$] but not [*pin*$^{-}$] strains suggests that the *in vivo* propagation of [*PIN*$^{+}$] is mediated by an aggregated form of Rnq1p.[6,7,30,35] However, a direct demonstration that Rnq1p aggregates are responsible

for [*PIN*$^+$] propagation, requires that *in vitro*-made Rnq1p aggregates exhibit [*PIN*$^+$]-specific infection ability.

Here, we establish a direct correspondence between *in vitro*-made amyloid-like aggregates of Rnq1p and the *in vivo* [*PIN*$^+$] propagation vehicle. This establishes the 'protein-only' nature of [*PIN*$^+$] and provides the ultimate 'prion-proof' for [*PIN*$^+$].

Results

Infectivity of crude cell extracts

The experimental design to assay for [*PIN*$^+$] infection by transforming [*PIN*$^+$]-specific seeds into [*pin*$^-$] yeast is outlined in Figure 1.

Spheroplasts of [*pin*$^-$][*psi*$^-$] yeast, which are auxotrophic for leucine, were co-transformed with crude cell extracts from different variants of [*PIN*$^+$] together with the *LEU2* plasmid pGALSUP35. Leu$^+$ transformants were tested for the presence of [*PIN*$^+$] by over-expressing Sup35p to test for induction of [*PSI*$^+$]. [*PSI*$^+$] induction, which indicated the presence of [*PIN*$^+$], was observed in 3–12 % of the Leu$^+$ transformants (Figure 2(a)). [*PSI*$^+$] induction was assayed by growth on medium lacking adenine (SC-Ade) and confirmed by guanidine curability (see Materials and Methods). In addition, the transformants that allowed [*PSI*$^+$] induction were also confirmed to be [*PIN*$^+$] by fluorescent decoration with Rnq1-GFP protein expressed using pID116. For this, the candidates were transformed with pID116 and grown in 50 μM $CuSO_4$ to induce Rnq1-GFP expression. As documented previously,[30] fluorescent Rnq1-GFP dots were observed only in those transformants that allowed [*PSI*$^+$] induction but not in [*pin*$^-$] controls.

[*PIN*$^+$] transformants induced [*PSI*$^+$] with an efficiency that was characteristic of the variant used to obtain the crude cell extract used in the transformation, indicating variant-specific infection (Figure 2(a)). Also, the low, medium and very high [*PIN*$^+$] transformants exhibited mostly a single Rnq1-GFP dot per cell whereas, the high [*PIN*$^+$] transformants prevalently contained multiple Rnq1-GFP dots per cell (data not shown).

No [*PIN*$^+$] transformants appeared in controls when crude cell extract from [*pin*$^-$] yeast was used in the transformation. Lack of [*PIN*$^+$] colonies upon transformation with crude cell extract from [*pin*$^-$] yeast shows that unlike Ure2p,[12] Rnq1p present in the prion-free cell extract does not spontaneously convert to a prion-prone conformation at a detectable rate. Cellular contamination in the extracts used was not the source of the [*PIN*$^+$] transformants recovered, because no viable cells were detected when the extracts were spread alone on complete medium. Also, no colonies were obtained on medium selective for plasmid transformants (Sorb-Leu) when only the crude cell extracts, without any added spheroplasts, were used in the transformation mixture.

When crude cell extract from [*RPS*$^+$] bearing yeast was used to infect a [*rps*$^-$] host, in a co-transformation experiment with pGALSUP35, [*RPS*$^+$] transformants were observed in 20% of the Leu$^+$ transformants (Figure 2(b)). No [*RPS*$^+$] transformants were obtained in controls, when crude cell extract from [*rPS*$^-$] yeast was used.

Aggregation of Rnq1p-(132–405)

Here, we examined the ability of a His-tagged fragment (aa: 132–405) of wild-type Rnq1p to form infectious amyloid aggregates *in vitro*. This fragment was chosen because it contained the amino acids 153–405, that were previously shown to be sufficient for propagation *in vivo* as the [*RPS*$^+$] prion when fused to MC region of Sup35p.[6] Full length recombinant Rnq1p,

with a 3-aa N-terminal extension (MGS) and a 5-aa deletion (NNGNQN) at the extreme C-terminus,[23] was previously converted into amyloid-like structures *in vitro*.[6]

Recombinant Rnq1p-(132–405) aggregated with typical sigmoidal kinetics as detected by the binding affinity of the amyloid-specific thioflavin-T dye (Figure 3(a)).[36] The aggregation consisted of a lag phase followed by a cooperative conversion to the thioflavin-T reactive state. Unshaken samples had a lag time of about 40 h, whereas agitated samples aggregated after only a 10 h lag, when incubated at similar protein concentrations (Figure 3(a)). Studies on the aggregation of monomeric Sup35pNM have shown that the acceleration of aggregation with agitation is predominantly a result of the fragmentation of large fibers generating new reactive ends thereby facilitating faster recruitment of monomers into new fibers.[37] The aggregation of Rnq1p-(132–405) was also accelerated when the protein concentration was increased, as observed by a decrease in the lag time. At a protein concentration of 25 μM, the lag time was about 25 h whereas, it decreased to about 10 h at 110 μM. No significant reduction in the lag time was observed above 110 μM up to 190 μM (Figure 3(b)).

When pre-formed aggregates were sonicated and added to monomeric Rnq1p-(132–405) incubated in the aggregation buffer, the lag preceding the aggregation was eliminated (Figure 3(c)). Even a seed concentration of only 1% (v/v) eliminated the lag. However, a lower initial rate of polymerization was observed when 1% of seed was used compared to higher seed levels. Possibly, the number of reactive ends is limiting at the 1% seed level and once these seeds mature and grow longer, they are subsequently fragmented by agitation thereby generating reactive ends sufficient in number to sustain the logarithmic rate of aggregation.[37] Noticeably, upon completion of the process, a higher thioflavin-T fluorescence intensity was in the seeded reactions compared to the unseeded aggregation, probably due to formation of more number of amyloid aggregates of relatively shorter sizes, and therefore with increased accessibility to thioflavin-T.

Aggregation of Rnq1p-(132–405) was substantially accelerated at 35° and 45 °C compared to 27 °C. At 70 μM, the lag time followed the order of 27 °C >35 °C >45 °C (Figure 3(d)). However, at 110 μM, the lag was similar at 35° and 45°C although less than at 27 °C.

Rnq1p-(132–405) aggregates have an amyloid-like stable conformation

As found previously for *in vitro*-made fibers of Sup35pN[13] and many other amyloidogenic proteins,[38,39] the *in vitro*-made aggregates of Rnq1p-(132–405), exhibited a single negative peak at 218 nm in the far-UV circular dichroism (CD) spectrum thereby suggesting that the protein conformation is largely β-sheet (Figure 4(a)). The non-aggregated stock protein stored in 9M urea exhibited a negative peak at around 205 nm in the far-UV CD spectrum which is indicative of an abundance of random coil conformation.

The *in vitro*-made aggregates, once polymerized were resistant to complete dis-aggregation by urea and their self-seeding ability was not completely lost even upon exposure to 8M urea (data not shown). However, the aggregates were dis-assembled upon exposure to increasing concentrations of GuHCl as monitored by their binding affinity to thioflavin-T (Figure 4(b)). Upon 90 min incubation at room temperature in 3M GuHCl, the ability of aggregates to bind thioflavin-T was comparable to the un-polymerized protein. Disruption of the *in vitro*-made amyloid aggregates of Sup35pNM has been reported at similar GuHCl concentrations.[40]

As seen previously for the *in vitro*-made amyloid fibers of Sup35pN,[13] *in vivo* prion aggregates of Sup35p from *Pichia methanolica*,[41] and also for the *in vitro*-made amyloid fibers of Ure2p,[15] the *in vitro*-made aggregates of Rnq1p-(132–405) were more resistant to proteinase K digestion than the soluble protein (Figure 4(c)).

Previous studies have shown the presence of high molecular weight SDS-stable aggregates, in cell extracts of [*PSI*$^+$][42] and [*PIN*$^+$] yeast,[35] as monitored by semi-denaturing agarose gel electrophoresis. When *in vitro*-polymerized Rnq1p-(132–405) aggregates were subjected to similar electrophoresis, high molecular weight SDS-stable aggregates were visualized, whereas the non-polymerized protein was mostly monomeric (Figure 4(d)). This finding is an indication of similarity between *in vitro*-made amyloid aggregates of Rnq1p-(132–405) and the *in vivo* self-propagating [*PIN*$^+$] aggregates. Therefore, these aggregates were further examined for their [*PIN*$^+$]-specific infectious ability, since the amyloid fibers of fragments of Sup35p and Ure2p are infectious.

Amyloid aggregates of Rnq1p-(132–405) are infectious

In vitro-polymerized amyloid aggregates of Rnq1p-(132–405), mixed with crude cell extract from [*pin*$^-$][*psi*$^-$] cells, were introduced into [*pin*$^-$][*psi*$^-$] yeast along with pGALSUP35 by transformation (Figure 1). Successful [*PIN*$^+$] infection was scored by the ability of over-expression of Sup35p to induce the *de novo* appearance of [*PSI*$^+$] in Leu$^+$ transformants. [*PSI*$^+$] induction was scored by increased translational read-through of the *ade1-14* nonsense mutation detected by growth on SC-Ade and was confirmed by guanidine curability. Since the rate of amyloid aggregation of Rnq1p-(132–405) was markedly different at 27° and 45 °C (Figure 3(d)), we infected [*pin*$^-$][*psi*$^-$] yeast with 300 μg of Rnq1p-(132–405) polymerized at these two temperatures to determine if these aggregates exhibit any difference in [*PIN*$^+$]-specific infectivity. Both caused [*PIN*$^+$] infection and with comparable frequencies of around 6–7% (Table 1). In both cases, mixtures of [*PIN*$^+$] variants were observed (data not shown).

The presence of [*PIN*$^+$] in transformants that exhibited [*PSI*$^+$] inducibility was further confirmed by decorating the *in vivo* Rnq1p aggregates with Rnq1-GFP protein expressed from pID116. These [*PIN*$^+$] transformants showed a mixture of cells bearing both single and multiple Rnq1-GFP fluorescent dots per cell (data not shown). In controls, no Rnq1-GFP fluorescent dots were observed when the Leu$^+$ transformants that were scored as [*pin*$^-$] because they did not show [*PSI*$^+$] inducibility, were used. No [*PSI*$^+$] induction was observed in a control where the [*pin*$^-$][*psi*$^-$] host was transformed only with the crude cell extract without any Rnq1p-(132–405) aggregates (Table 1). This indicates that the observed [*PIN*$^+$] infection was due to the *in vitro*-made Rnq1p-(132–405) amyloid aggregates.

In simultaneously performed control experiments, when the Rnq1p-(132–405) aggregates polymerized at 27 °C and subjected to overnight digestion with an excess of proteinase K were used as seed, in a mixture with cell extracts from [*pin*$^-$][*psi*$^-$] yeast, no [*PIN*$^+$] transformants were obtained. Also, no [*PIN*$^+$] infection was obtained when the non-aggregated stock protein was used as seed in the infection assay (Table 1). Taken together, these control experiments show protein-basis of infectivity and show a dependence of the infectivity on the amyloid aggregates of Rnq1p-(132–405).

We also succeeded in demonstrating the infectivity of Rnq1p-(132–405) aggregates by modifying the 'traditional' transformation procedure, used for [*PSI*$^+$] and [URE3] infectivities,[11,12] by substituting PEG 3350 for PEG 8000. Using this method, in the absence of any added crude cell extract, about 4% of the plasmid transformants showed [*PIN*$^+$] infection (Table 1). Again, similar [*PIN*$^+$] infection frequencies were obtained whether the aggregates were made at 27° or 45 °C. Also, in both cases, the obtained [*PIN*$^+$] transformants were mixtures of [*PIN*$^+$] variants (data not shown). The [*PIN*$^+$] infection frequency obtained this way was less than when the transformation was carried out after pre-mixing with [*psi*$^-$][*pin*$^-$] crude cell extract (Table 1). The ability of the *in vitro*-made amyloid aggregates of Rnq1p-(132–405) to induce [*PIN*$^+$] when transformed without pre-mixed crude cell extract clearly shows that the aggregates of Rnq1p-(132–405) are the infectious entities.

Discussion

In this study, we successfully generate *in vitro* infectious amyloid aggregates of a Gln/Asn-rich fragment of Rnq1p that are [*PIN*$^+$]-specific.

We first show that the crude cell extracts from variants of [*PIN*$^+$] transform a [*pin*$^-$] host in a 'variant-specific' fashion, thereby confirming their infectious nature. Also, the crude cell extracts from [*RPS*$^+$] yeast, which contain prion conformation of the chimeric Rnq1p-(153–405)-MC protein,[6] were found to be infectious to [*rPS*$^-$] host cells. Consistent with previous *in vivo* results, where both [*pin*$^-$] and [*RPS*$^+$] meiotic progeny were obtained from a diploid made by mating [*RPS*$^+$] and [*pin*$^-$] haploids,[6] we likewise find that [*RPS*$^+$] does not infect [*pin*$^-$] when the crude cell extract from [*RPS*$^+$] yeast is transformed into a [*pin*$^-$] yeast, nor were [*PIN*$^+$] extracts able to infect a [*rPS*$^-$] host (data not shown). The reason for this incompatibility of [*PIN*$^+$] and [*RPS*$^+$] is not known. However, if the M region of Sup35p contributes to the self-replicating structural core of [*RPS*$^+$], as it appears to in [*PSI*$^+$],[32,33] the M domain may cause a steric hindrance preventing Rnq1p from joining Rnq1p-(153–405)-MC aggregates.

The relationship between *in vitro*-made amyloid aggregates from certain prion proteins and the *in vivo* self-replicating prion aggregates has been debated,[43] although considerable evidence supports a direct correspondence for [*PSI*$^+$] and [URE3].[10–12] Here we set out to establish a direct parallel between the *in vitro*-made amyloid aggregates of the recombinant Rnq1p-(132–405) and the propagation elements of [*PIN*$^+$]. Thus, we aggregated a recombinant fragment of Rnq1p containing aa 132–405. The aggregation, followed by thioflavin-T binding, exhibited typical trends expected of amyloid fiber formation: cooperative conversion to thioflavin-T reactive state after a preceding lag period and self-seeding ability as observed by the elimination of the lag upon the addition of briefly sonicated pre-formed aggregates. The elimination of the lag, by addition of the sonicated pre-formed *in vitro*-made aggregates, strongly suggests that they are ordered polymers rather than amorphous aggregates. We also observed an acceleration of the aggregation with temperature. Successful amyloid aggregation requires first that the amino acids critical for making intermolecular contacts are appropriately exposed and establish specific intermolecular contacts with a second molecule and then with many more molecules.[44] Possibly, the increased protein concentration and temperature enhance the frequency of conveyance of amyloid-prone conformation to the monomeric Rnq1p-(132–405) and hence association into amyloid polymers.

The *in vitro*-made aggregates of Rnq1p-(132–405) exhibited a predominance of β-sheet-rich conformation and showed resistance to complete dis-aggregation by urea, and the ionic detergent SDS and digestion by sproteinase K. These characteristics indicate the amyloid-like nature of these aggregates.

Several initial attempts to obtain [*PIN*$^+$] infection, by transforming the *in vitro*-made amyloid aggregates of Rnq1p-(132–405) into [*pin*$^-$][*psi*$^-$] yeast with the original Tanaka *et al* method[11] used for [*PSI*$^+$] infection, failed. However, we did obtain [*PIN*$^+$] infection by pre-mixing these Rnq1p-(132–405) amyloid aggregates with crude cell extract from [*pin*$^-$][*psi*$^-$] yeast, although cell extract did not influence the infectivity of amyloid fibers of Ure2p.[12]

To definitively demonstrate that the *in vitro*-made aggregates of Rnq1p-(132–405) alone are sufficient for infection, we tried modifications of the transformation method to find conditions where [*PIN*$^+$]-specific infectivity could be obtained without pre-mixing the aggregates with crude cell extracts. Indeed, we accomplished this by replacing PEG 8000 with PEG 3350 in the transformation buffer (Table 1). A possible reason for this success may be the difference in the protein precipitating abilities of PEG 3350 and PEG 8000 polymers. The potential of polyethylene glycol to precipitate a protein is known to increase with increasing molecular

weight of the PEG polymer.[45] It is therefore possible that the lack of the infectivity in the presence of PEG 8000 buffer, when no cell extract from [*pin*$^-$][*psi*$^-$] is added, might be a reflection of the coalescence of the amyloid aggregates to molecular sizes larger than can be delivered into the yeast cell. Possibly, the addition of crude cell extracts to the Rnq1p-(132–405) aggregates prevents the seeds from precipitating into large aggregates thereby increasing the [*PIN*$^+$] infection frequency to detectable levels.

Although, the observed rates of the *in vitro* aggregation of Rnq1p-(132–405) at 27° and 45° C were different, when used to infect [*pin*$^-$] yeast, these aggregates did not exhibit any predisposition toward inducing any specific [*PIN*$^+$] variant. In both cases, and irrespective of whether infection was obtained with or without pre-mixed crude cell extracts, mixtures of [*PIN*$^+$] variants resulted (data not shown). On the contrary, in a previous study, Sup35pNM amyloid fibers made at different temperatures, when transformed into [*psi*$^-$] yeast, showed preferential induction of certain [*PSI*$^+$] variants.[11]

The discovery of [*PIN*$^+$] as a [*PSI*$^+$]-induction facilitating prion started a new concept of *in vivo* 'prion-prion' interactions[7] although, at present all the three yeast prions are known to interact. The demonstration of the amyloid basis of [*PIN*$^+$] propagation in this study, and previously of [*PSI*$^+$][10,11] and [URE3],[12] supports the hypothesis that the yeast prions interact *in vivo* through their corresponding amyloids.

These results establish that Rnq1p aggregates form the self-propagating structural core for [*PIN*$^+$] transmission and definitively place [*PIN*$^+$] in the category of amyloid-based prions. The study also shows that complete Rnq1p is not essential for obtaining [*PIN*$^+$] infection and supports the general modular architecture of prion proteins, which advocates that only the prion domains are sufficient for induction and propagation of prions.[46] The infection of [*pin*$^-$] yeast with the *in vitro*-made amyloid aggregates of Rnq1p-(132–405) alone shows that the [*PIN*$^+$] infectivity is 'protein-only'. Further, the 'protein-only' transmission of [*PIN*$^+$] explicitly provides proof of the prion-hypothesis for [*PIN*$^+$].

Materials and Method

Yeast culture media

Standard yeast media and cultivation conditions were used.[47] [*PSI*$^+$] induction was scored on synthetic complete dextrose medium (SC) lacking adenine (SC-Ade). In the prion co-transformation assays, the plasmid transformants were selected on Sorb-Leu (SC-Leu with 3% glucose + 0.9 M sorbitol) and were then patched on 'master' plates of SC lacking leucine (SC-Leu). [*PSI*$^+$] curing was achieved on complex glucose medium (YPD) containing 4 mM guanidine hydrochloride (YPD + 4mM GuHCl). Over-expression of pGALSUP35 was obtained on SGal-Leu plates (synthetic complete medium lacking leucine and containing 2% galactose and 1% raffinose as the carbon sources). 50 μM $CuSO_4$ was used to express *CUP1* promoter driven genes.

Yeast strains and plasmids

The [*psi*$^-$][*pin*$^-$] and [*PIN*$^+$] variant bearing yeast strains[30] were derivatives of 74-D694 (*MAT***a** *ade1-14 his3-200 ura3-52 leu2-3, 112 trp1-289*). The [*RPS*$^+$] and [*rPS*$^-$] yeast,[6] where the genomic *SUP35* is replaced by *RMC* (a fusion of the *SUP35* promoter, the region of *RNQ1* encoding aa 153–405 (R) and the region of *SUP35* encoding aa 124–685 (MC)) and *RNQ1* is disrupted by a *KANMX*$_4$ cassette, were also derivatives of 74-D694 (kind gift of Susan Lindquist, Whitehead Institute).

The plasmid pGALSUP35 (*LEU2 CEN GAL1p::SUP35*)[26] was used in prion co-transformation experiments where it was also utilized for the *de novo* induction of [*PSI*$^+$]. The

Rnq1-GFP fusion protein was expressed using pID116 (*HIS3 CUP1p::RNQ1GFP*) (a kind gift of Irina Derkatch, New York University). pID116, which contains the wild-type *RNQ1* sequence, was also used as template for PCR amplification of a C-terminal fragment of *RNQ1* encoding a Q/N rich region (aa: 132–405) using primers 1: CGTCATATGGGCCAAAG TATG GGTGCTTCC and 2: CTTTGGGGCCCTACCGCGGGTAGCGGTT. The amplified product was then cloned as an *NdeI+ApaI* restriction fragment into a recombinant expression vector, pJC45[48] (a kind gift of Joachim Clos, Bernhard Nocht Institute) to obtain a plasmid named pHis$_{10}$-Rnq1PD.

Scoring for [*PSI*$^+$] and [*PIN*$^+$]

[*PSI*$^+$] was scored as described.[49] The assay for [*PSI*$^+$] in 74-D694 derivatives uses a premature nonsense mutation (*ade1-14*) in *ADE1*. In [*psi*$^-$] cells, where the translation termination factor Sup35p (eRF3) is functional, only truncated translation product of *ADE1* is obtained due to efficient translation termination at the premature stop codon in *ade1-14*. Such cells are unable to grow on SC-Ade and are red on YPD. Whereas, in [*PSI*$^+$] cells, Sup35p is partially inactivated due to its sequestration into amyloid aggregates resulting in read-through of the *ade1-14* mutation (nonsense suppression) thereby enabling cells to grow on SC-Ade. Such Ade$^+$ colonies are white or pink on YPD. *De novo* induction of [*PSI*$^+$] was achieved by over-expression of Sup35p from pGALSUP35 by growing cells on SGal-Leu (48 h). The cells were assayed for [*PSI*$^+$] by growth on SC-Ade. The Ade$^+$ white yeast were further confirmed to be [*PSI*$^+$] by guanidine curability as observed by the appearance of red colonies on YPD when streaked from YPD + 4mM GuHCl.[50,51]

The presence of [*PIN*$^+$] was scored by the inducibility of [*PSI*$^+$] upon over-expression of Sup35p.[7,26] Furthermore, [*PIN*$^+$] yeast were transformed with pID116 and the presence of Rnq1p aggregates *in vivo* was confirmed by fluorescent decoration of the aggregates. The Rnq1-GFP fusion protein was expressed from pID116 by growth for 5 h in liquid SC-His + 50 μM $CuSO_4$.[30] Variants of [*PIN*$^+$] were distinguished by the efficiency of [*PSI*$^+$] induction as described previously.[30] Briefly, cells bearing pGALSUP35 were grown as patches on SGal-Leu for 48 h, diluted in water and ~1000 cells were spotted on SC-Ade to score for [*PSI*$^+$]. The low, medium, high and very high [*PIN*$^+$] yeast respectively exhibit low to very high levels of growth on SC-Ade. Also, when pID116 is expressed, high [*PIN*$^+$] shows mostly multiple fluorescent dots per cell whereas, the low, medium and very high [*PIN*$^+$] prevalently exhibit a single dot per cell.[30]

Preparation of crude cell extracts from yeast

Crude cell extracts were obtained in STC (1M sorbitol, 10 mM $CaCl_2$, 10 mM TrisHCl pH 7.5) buffer containing a protease inhibitor cock-tail (Sigma Chemicals) at 4 °C by vortexing with 0.5 mm glass beads and then pre-clearing twice before use, first at 3000 rpm and then at 3300 rpm.

Preparation of yeast spheroplasts

The [*psi*$^-$][*pin*$^-$] or [*rPS*$^-$] yeast, respectively used as hosts for assaying infectivity of [*PIN*$^+$] and [*RPS*$^+$]-specific seeds, were spheroplasted using lyticase as previously described.[11] The spheroplasts were maintained in STC buffer at 4 °C and used the same day.

Prion transformation into yeast spheroplasts

The infectious nature of crude cell extracts either from [*PIN*$^+$] or [*RPS*$^+$] yeast and the *in vitro*-made amyloid aggregates of recombinant Rnq1p-(132–405), was assayed in co-transformation experiments with pGALSUP35, by appropriate modifications in the method described for [*PSI*$^+$] infectivity.[11] The final transformation mixture contained either the PEG

8000 (20% PEG 8000, 10 mM $CaCl_2$, 10 mM Tris pH 7.5)[11] or PEG 3350 buffer (25% PEG 3350, 10 mM $CaCl_2$, 10 mM Tris pH 7.5). The scheme for the [*PIN*$^+$] infection assay is outlined in Figure 1. After selection for the plasmid on Sorb-Leu, the transformants were patched on SCLeu and then assayed for [*PIN*$^+$] infection by [*PSI*$^+$] inducibility.

Protein expression and purification

The plasmid pHis$_{10}$-Rnq1PD was transformed into *E. coli BL21AI* (Invitrogen). Protein over-expression was achieved at 37 °C by induction with 1 mM IPTG together with 0.15 % L (+) arabinose. The expressed recombinant protein was purified under denaturing conditions in 9 M urea using Ni-NTA agarose affinity column chromatography (Qiagen) as per the recommendations of the manufacturer. The protein concentration was estimated using a calculated extinction coefficient of $\varepsilon_{280}{}^{1\%}$=5.1 and aliquots of 100 µl were stored at −70 °C for further use.

Kinetics of *in vitro* aggregation of Rnq1p-(132–405)

Rnq1p-(132–405) stock, stored in 9M urea, was diluted to 110 µM in 100 mM phosphate buffer pH 7.3 containing 4M urea and 1M NaCl (aggregation buffer). 5mM thioflavin-T dye stock was added to 130 µl of this mixture to get 100 µM final and transferred into a 96 well microplate. The microplate was incubated overnight in a Molecular Devices, Spectramax-M2 microplate reader pre-equilibrated at 27°, 35° or 45 °C. The plate was agitated intermittently for 30 sec every 3 min and amyloid aggregation was monitored by thioflavin-T binding, assayed by recording the increase in fluorescence at 382 nm upon excitation at 350 nm.[36] To monitor the effect of agitation on the aggregation kinetics, the samples were incubated either without any agitation or with agitation as above.

The ability of *in vitro*-made aggregates to seed polymerization of non-aggregated Rnq1p-(132–405) was examined by adding increasing concentrations (1–8 %, v/v) of briefly sonicated pre-formed aggregates to the aggregation mixture.

Characterization of the Rnq1p-(132–405) aggregates

Secondary structure—Pre-formed *in vitro*-made Rnq1p-(132–405) aggregates were briefly sonicated and diluted 10 fold in fresh aggregation buffer to a final concentration of 10 µM before recording far-UV circular dichroism spectrum from 260 nm to 195 nm on a Jasco J710 spectropolarimeter using a 1 mm path-length quartz cuvette at a scan speed of 50 nm per min. The spectrum was also recorded for the stock protein stored in 9M urea after diluting it to 10 µM in fresh 9M urea stock. All spectra were corrected for base line contributions and expressed as mean residue ellipticities $[\theta]_{mrw}$.

Proteinase K resistance—Relative resistance of the Rnq1p-(132–405) aggregates to digestion by proteinase K was compared to that of the soluble protein. 80 µM of aggregates or the soluble protein in 4M urea were incubated at 37 °C for 30 min in the presence of increasing concentrations of proteinase K (0.2 to 0.8 µg/ml). The reaction was terminated by the addition of PMSF to a final concentration of 5 mM. 12 M urea was added to equal aliquots of the reaction mixture to get a final concentration of 8M prior to electrophoresis on a 12 % SDS-polyacrylamide gel under reducing conditions and the protein was stained with Coommassie R-250 dye.[52]

SDS stability—The *in vitro*-made Rnq1p-(132–405) aggregates (30 µg) were assayed for their stability against 2% SDS (Sodium dodecyl sulfate) by incubation at room temperature with a non-reducing sample buffer, which contains 2% SDS,[52] for 10 min and electrophoresis in a 1.5% agarose gel as described for [*PIN*$^+$] cell extracts.[35] The protein was electro-blotted

to a PVDF membrane and probed using rabbit anti-Rnq1 primary antibody-type II (a kind gift of Susan Lindquist, White Head Institute).

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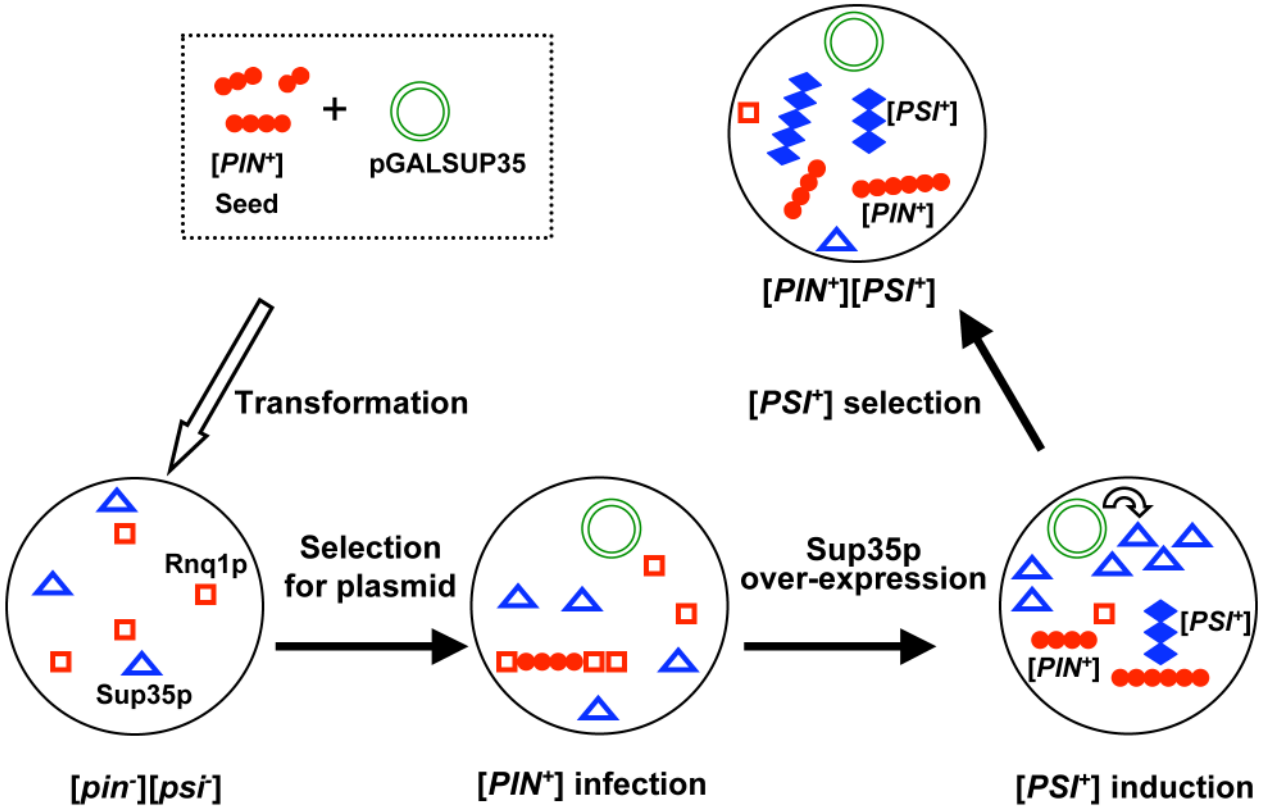

Figure 1. Scheme of *in vitro* transformation of [*pin*$^{-}$] yeast

Amyloid aggregates of Rnq1p-(132–405) or crude cell extracts from yeast bearing variants of [*PIN*$^{+}$] were introduced by co-transformation into [*pin*$^{-}$][*psi*$^{-}$] cells along with the *LEU2* plasmid pGALSUP35. Plasmid bearing transformants were selected on leucine-less medium (see Materials and Methods). Some of the Leu^{+} transformants also took up the infective [*PIN*$^{+}$]-specific Rnq1p aggregates which converted these cells to [*PIN*$^{+}$] by sequestering the endogenous Rnq1p into prion aggregates. We used the fact that [*PIN*$^{+}$] facilitates the appearance of [*PSI*$^{+}$] to score the transformants for [*PIN*$^{+}$]. The Leu^{+} transformants were grown for 48 h on plasmid selective galactose medium to over-express Sup35p, which causes the appearance of [*PSI*$^{+}$] in the presence of [*PIN*$^{+}$]. Induction of [*PSI*$^{+}$] as a measure of [*PIN*$^{+}$] infection was assessed by growth on adenine-less medium (SC-Ade) and confirmed by curability by GuHCl (see Materials and Methods).

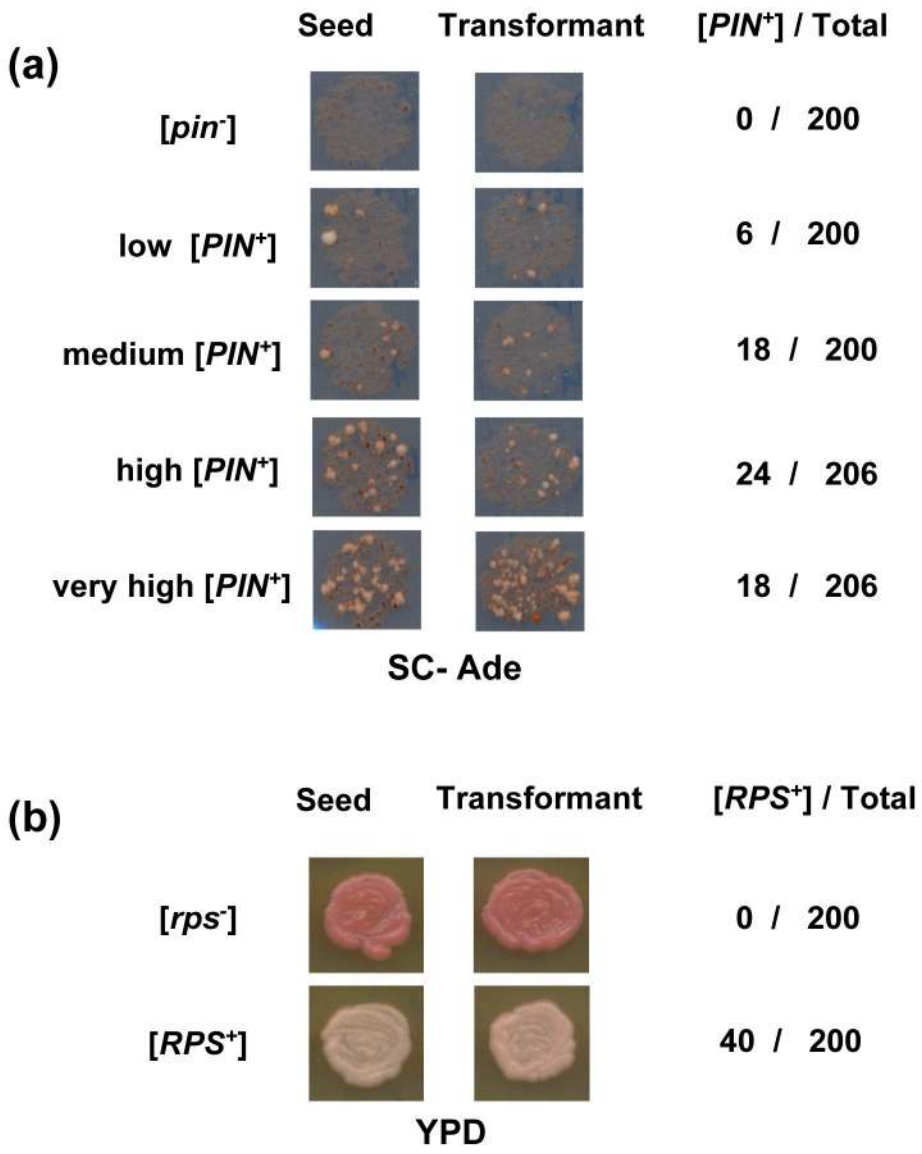

Figure 2. Variant-specific infectivity of crude cell extracts from [*PIN*$^+$] and [*RPS*$^+$] yeast

(A) Crude cell extracts containing 300 μg protein from yeast bearing the four [*PIN*$^+$] variants (low, medium, high or very high) or from [*pin*$^-$] yeast were introduced into [*pin*$^-$][*psi*$^-$] cells via co-transformation with pGALSUP35. Plasmid transformants (Transformant) and the yeast from which the extracts used as seed were made (Seed) were assayed for [*PIN*$^+$] by determining if [*PSI*$^+$] could be induced by Sup35p over-expression. [*PIN*$^+$] variants were distinguished by the efficiency with which they promote [*PSI*$^+$] appearance as detected by growth on adenine-less medium (SC-Ade).[30] **(B)** Crude cell extracts from [*RPS*$^+$] or [*rPS*$^-$] yeast containing 300 μg protein were co-transformed with pGALSUP35 into [*rPS*$^-$] yeast and Leu$^+$ transformants (Transformant) and the yeast from which the extracts used as seed were made (Seed) were assayed for [*RPS*$^+$] by patching single colonies on complex medium (YPD) and examining color. [*RPS*$^+$] and [*rPS*$^-$] yeast are respectively white and red.

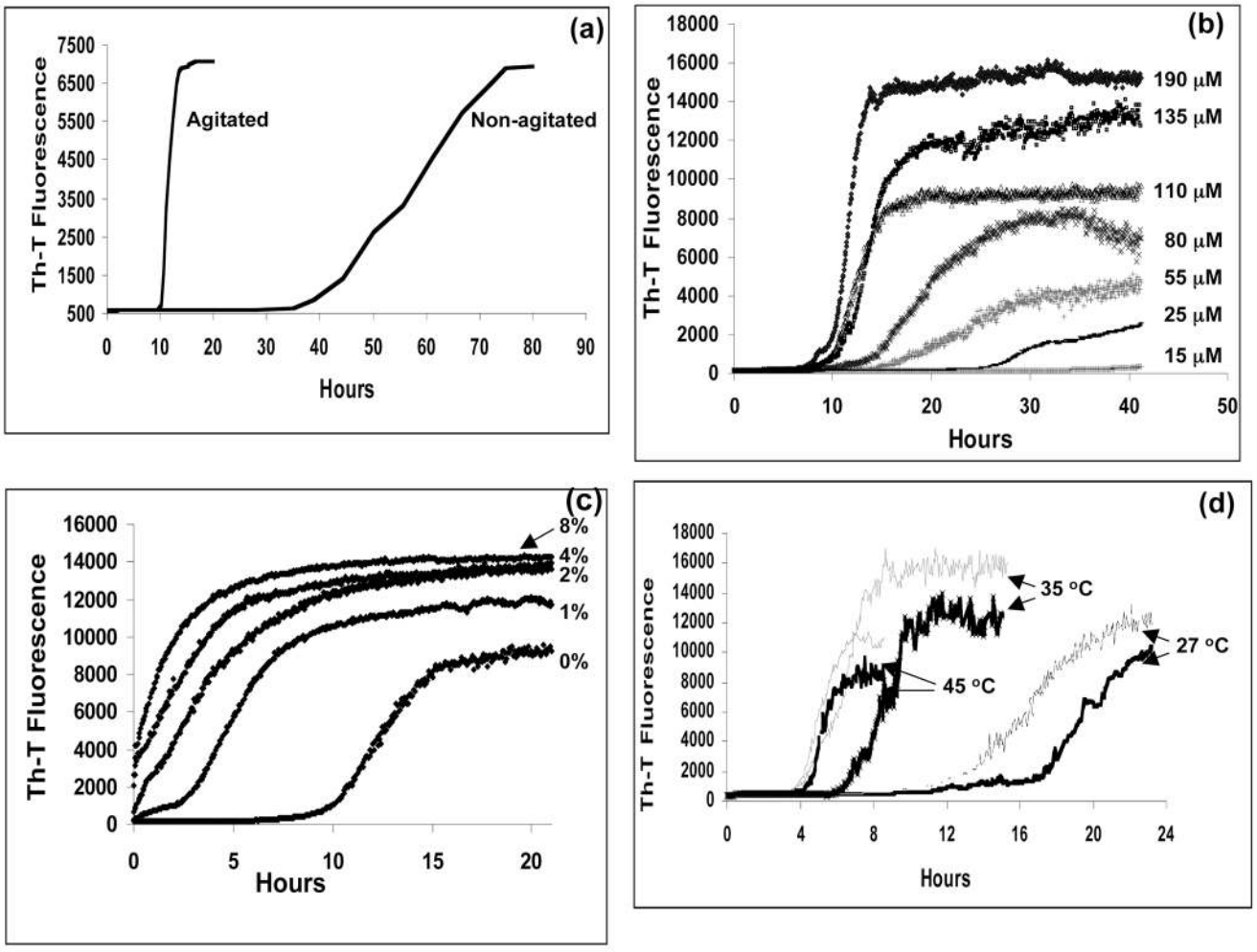

Figure 3. Kinetics of *in vitro* aggregation of recombinant Rnq1p-(132–405)

(A) Effect of agitation on the rate of aggregation of 110 μM Rnq1p-(132–405) at 27 °C measured by thioflavin-T dye binding. Samples were incubated either without or with 30 sec agitation every 3 min. **(B)** Effect of protein concentration on the rate of Rnq1p-(132–405) aggregation monitored with agitation at 27 °C **(C)** Effect of pre-formed seed concentration on the aggregation kinetics of 110 μM Rnq1p-(132–405) agitated at 27 °C **(D)** Effect of increasing temperature on the rate of aggregation of Rnq1p-(132–405) monitored with agitation. Thick lines represent 70 μM and the thin lines indicate 105 μM of the protein.

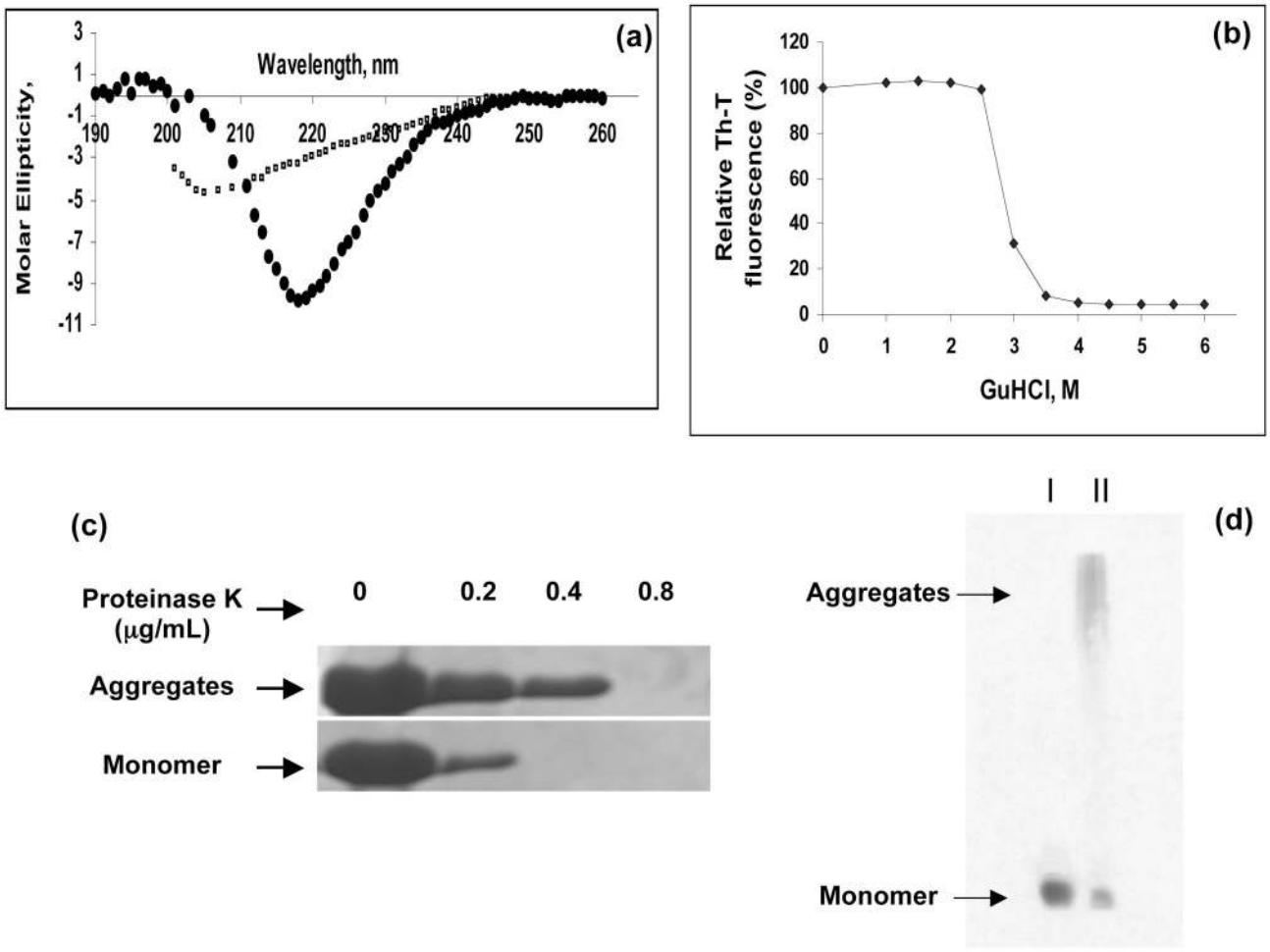

Figure 4. Characterization of *in vitro*-made aggregates (27 °C) of Rnq1p-(132–405)

(A) Secondary structure in sonicated Rnq1p-(132–405) aggregates (●) monitored by far UV circular dichroism compared with non-aggregated protein maintained in 9M Urea (□). **(B)** Stability of Rnq1p-(132–405) aggregated (55 μM) to increasing GuHCl concentration 22 incubated for 90 min at room temperature. **(C)** Proteinase K digestion of the *in vitro*-made Rnq1p-(132–405) aggregates compared to the monomeric protein, incubated at 37 °C for 30 min. **(D)** Western blot showing stability of the aggregates of Rnq1p-(132–405) to 2% SDS monitored by electrophoresis on 1.5% agarose gel and probed by anti-Rnq1 type-II antibody. Lane I, non-aggregated protein; Lane II, *in vitro*-made aggregates.

Table 1

'Protein-only' infection by Rnq1p-(132–405) aggregates

Seed	Added cell extract	PEG buffer	Number of plasmid transformants examined[a]	Number of [*PIN*$^+$][b]	Infection frequency
Rnq1p-(132–405) aggregates(27 °C)	[*pin*$^-$][*psi*$^-$]	8000	238	15	6 %
Rnq1p-(132–405) aggregates(45 °C)	[*pin*$^-$][*psi*$^-$]	8000	234	16	7 %
None	[*pin*$^-$][*psi*$^-$]	8000	256	0	0 %
Proteinase K over-digested[c]	[*pin*$^-$][*psi*$^-$]	8000	220	0	0 %
Non-aggregated Rnq1p-(132–405)	[*pin*$^-$][*psi*$^-$]	8000	189	0	0 %
Rnq1p-(132–405) aggregates(27 °C)	NONE	3350	248	10	4 %
Rnq1p-(132–405) aggregates(45 °C)	NONE	3350	254	9	3.5 %

[a] 300 μg of in vitro made amyloid aggregates of Rnq1p-(132–405) were co-transformed into [*psi*$^-$][*pin*$^-$] yeast with pGALSUP35

[b] Leu$^+$ transformants were examined for [*PIN*$^+$] infection by assaying [*PSI*$^+$] inducibility

[c] Rnq1p-(132–405) aggregates made at 27 °C were digested overnight with excess of proteinase K.