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Human cellular prion protein interacts directly with clusterin protein

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Prion protein is a glycosyl-phosphatidyl-inositol anchored glycoprotein localized on the surface and within a variety of cells. Its conformation change is thought to be essential for the proliferation of prion neurodegenerative diseases. Using the yeast two-hybrid assay we identified an interaction between prion protein and clusterin, a chaperone glycoprotein. This interaction was confirmed in a mammalian system by *in vivo* co-immunoprecipitation and *in vitro* by circular dichroism analysis. Through deletion mapping analysis we demonstrated that the α subunit, but not the β subunit, of clusterin binds to prion and that the C-terminal 62 amino acids segment of the putative α helix region of clusterin is essential for the binding interaction. The full prion protein as well as the N-terminal section (aa 23-95) and C-terminal (aa 96-231) were shown to interact with clusterin. These findings provide new insights into the molecular mechanisms of interaction between prion and clusterin protein and contribute to the understanding of prion protein's physiological function.

INTRODUCTION

Prion diseases[1], also known as transmissible spongiform encephalopathies (TSEs), are a group of fatal neurodegenerative disorders that include Creutzfeldt-Jakob disease (CJD) and its variant form (vCJD) in humans[2], bovine spongiform encephalopathy (BSE) or mad cow disease in cattle, scrapie in sheep, and chronic wasting disease (CWD) in deer[3]. In afflicted organisms the central nervous system accumulates increased levels of a pathological isoform (PrP^{sc}) of the naturally occurring prion protein (PrP^c). PrP^{sc} is more resistant to degradation and forms aggregates and amyloid fibrils. The normal PrP^c protein contains mainly an α helix structure whereas in the PrP^{sc} isoform the β sheet conformation predominates. The mature human PrP^c contains amino acids (aa) 23-231 and is generated by a cleavage of the N-terminal signal peptide residues 1-22 and removal of the C-terminal hydrophobic peptide residues 232-253 in a transamidation reaction which attaches a glycosyl-phosphatidyl-inositol (GPI) moiety to a terminal serine amino acid of mature PrP^c. The GPI anchor attaches the PrP^c to cellular membranes throughout the cell including the cell surface outer membrane. Further post-translational modifications include glycosylation at two conserved asparagine sites[4] of the C-terminal PrP^c and result in three PrP^c glycoforms (unglycosylated, monoglycosylated, and diglycosylated).

PrP^c is widely expressed in many tissues, with the highest expression in the central nervous system (CNS) and the peripheral nervous system (PNS). Despite decades of research,

the physiological function of PrPc is still not fully elucidated. PrPc knockout mice[5, 6] and cow[7] develop normally, but are resistant to PrP^{Sc} infection in vivo and in vitro which demonstrates the essential role of PrPc in prion diseases. One approach to characterize the physiological role of the PrPc is to identify its ligands. Several studies have demonstrated with either the bacterial or yeast two-hybrid system (Y2H) that cellular chaperone proteins Hsp60[8], aB-crystalline[9] Rdj2[10], and other group proteins such as TREK-1[11], NRAGE[12], synapsin Ib, Pint1, Grb2[13], laminin receptor[14] and Bcl-2[15] are prion binding ligands. Based on these observations, certain functions of PrPc have been proposed. These include protection against apoptotic and oxidative stress, cellular binding and uptake of copper ions, transmembrane signaling formation and maintenance of synapses, and adhesion to the extracellular matrix[16].

In this study, we used a yeast two-hybrid system to demonstrate that clusterin, a chaperone protein, can directly interact with PrPc. These interactions were further supported by in vivo co-immunoprecipitation and in vitro circular dichroism (CD) analysis. Our study expands on previous findings of possible role of clusterin in TSEs [17-19], provides new insights into the molecular mechanisms of interaction between PrPc and its ligands and expands our understanding of PrPc's functions in normal physiological conditions.

MATERIALS AND METHODS

The Matchmaker Two-Hybrid System 3 (Clontech, Palo Alto, CA, USA) was used to detect protein-protein interactions. All primers and plasmids used in this study are listed in table 1 and 2, respectively.

Yeast two-hybrid prion plasmids

Bait prion plasmid pFX2

Human PrP^c encoding the mature prion amino acid 23-231 sequence was amplified by polymerase chain reaction (PCR) with primers FXF1 and FXR1 from human genomic DNA. The amplified PrP²³⁻²³¹ DNA was cloned into pGEM3Z vector (Promega, Madison, WI, USA), yielding the pFX1 plasmid. The PrP²³⁻²³¹ DNA was further cloned into pGBKT7, a GAL4 DNA-binding domain (DNA-BD) vector from Clontech, yielding the bait construct pFX2. This pFX2 was transformed into yeast *Saccharomyces cerevisiae* AH109 (MAT a) (Clontech), yielding the prion bait yeast AH109 [pFX2].

PrP²³⁻⁹⁵ plasmid pFX33

Plasmid pFX2 containing PrP²³⁻²³¹ was cut with PstI to get rid of the prion coding region 96-231. The resulting large fragment was self ligated to obtain the plasmid pFX33 which contained the prion N-terminal amino acids 23-95 (PrP²³⁻⁹⁵).

PrP⁹⁵⁻²³¹ plasmid pFX70

A fragment containing prion coding region C-terminal amino acids 95-231 was isolated from plasmid pFX2 cut with PstI. This PrP⁹⁵⁻²³¹ fragment was subsequently cloned into pGBKT7 cut with PstI to obtain the plasmid pFX70.

Yeast two-hybrid screen

The pray yeast *Saccharomyces cerevisiae* Y187 (MAT a) pretransformed with human adult brain cDNA library expressed as a fusion to the GAL4 activation domain (AD) in vector pGADT7-Rec (Clontech) was mated with the bait yeast AH109 [pFX2]. The resulting library mating diploid mixture was screened on synthetic dropout (SD) medium in the absence of four nutrients (leucine, tryptophan, adenine, and histidine) and further confirmed on X-a-Gal medium, indicator plate for MEL1 (a-galactosidase regulated by GAL4) response. The inserted cDNA fragments of positive clones from the human brain library screen were amplified directly from yeast colonies with PCR using pGADT7-Rec primer FXF3 and FXR3. The resulting PCR products were directly sequenced and compared to the DNA database at the National Center for Biotechnology Information using BLAST.

Yeast two-hybrid clusterin plasmids

Plasmid pGADT7 AD vector used for the yeast two-hybrid assay was from Clontech. Human clusterin plasmid (Clone: HsCD00000240) was from Dana-Farber/Harvard Cancer Center DNA Resource Core (Cambridge, MA, USA). StrataClone™ PCR Cloning Kit (pSC-A vector) from Stratagene (La Jolla, CA, USA) was used to clone PCR fragments.

Clusterin²¹⁵⁻⁴⁴⁹ plasmid pFX48

The pray plasmid pFX48 containing clusterin amino acids 215-449 (clusterin²¹⁵⁻⁴⁴⁹) insert was isolated from the yeast two-hybrid screen positive clone.

Clusterin a subunit plasmid pFX90

Clusterin a subunit (clusterin²²⁸⁻⁴⁴⁹) was amplified by PCR with primers FX25F and FX22R from a human clusterin plasmid and cloned to obtain the plasmid pFX89. Plasmid pFX89 was cut with BamHI and XhoI and the resulting clusterin a subunit was cloned into pGADT7 vector to get the plasmid pFX90.

Clusterin²⁷³⁻⁴⁴⁹ plasmid pFX64 and clusterin³⁰⁸⁻⁴⁴⁹ plasmid pFX73

Clusterin²¹⁵⁻⁴⁴⁹ plasmid pFX48 was cut with EcoRI or BglII to get rid of the coding region of clusterin 215-272 or 215-307. The resulting big fragments containing clusterin coding region 273-449 or 308-449 were self ligated to get the plasmid pFX64 or pFX73.

Clusterin³²⁰⁻³⁸¹ plasmid pFX97

Clusterin³²⁰⁻³⁸¹ was amplified by PCR with primers FX27F and FX29R from a human clusterin plasmid and cloned to obtain plasmid pFX95. Plasmid pFX95 was cut with BamHI and XhoI and the resulting Clusterin³²⁰⁻³⁸¹ was cloned into pGADT7 to obtain the plasmid pFX97. This Clusterin³²⁰⁻³⁸¹ contains a helix region predicted from software PredictProtein[20].

Clusterin³¹⁴⁻³⁴⁹ plasmid pFX92

Clusterin³¹⁴⁻⁴⁴⁹ was amplified by PCR with primer FX28F and FX28R from a human clusterin plasmid and directly cloned into pGADT7 to obtain the plasmid pFX92. This clusterin³¹⁴⁻⁴⁴⁹ contains a coiled-coil leucine zipper-like regions[21].

Clusterin³⁴⁹⁻³⁸¹ plasmid pFX110

Clusterin³⁴⁹⁻³⁸¹ was amplified by PCR with primer FX36F and FX29R from a human clusterin plasmid and cloned into pGADT7 to obtain the plasmid pFX110.

Clusterin³⁷⁵⁻⁴⁴⁹ plasmid pFX81

Clusterin³⁷⁵⁻⁴⁴⁹ was amplified by PCR with primer FX23F and FX22R from pFX64, and cloned to obtain the plasmid pFX79. Plasmid pFX79 was cut with BamHI and XhoI and the resulting Clusterin³⁷⁵⁻⁴⁴⁹ was cloned into pGADT7 vector to obtain the plasmid pFX81.

Clusterin β subunit plasmid pFX77

Clusterin β subunit clusterin²³⁻²²⁷ was amplified by PCR with primer FX21F and FX21R from a human clusterin plasmid and cloned to obtain the plasmid pFX75. Plasmid pFX75 was cut with BamHI and XhoI and the resulting clusterin β subunit was cloned into pGADT7 vector to obtain the plasmid pFX77.

Mammalian cell expression vectors, transfection, co-immunoprecipitation, gel electrophoresis and Western blot analysis

Plasmid pFX2 containing PrP²³⁻²³¹ was cut with SfiI+Sall, inserted into mammalian expression vector pCMV-HA (Clontech) cut with SfiI+XhoI to obtain the PrPc expression plasmid pFX43. Clusterin²⁷³⁻⁴⁴⁹ PCR product amplified by primers FX22F and FX22R from a human clusterin plasmid was cut with BamHI+XhoI, inserted into mammalian expression vector pCMV-Myc (Clontech) cut with BamHI+XhoI to obtain the clusterin expression plasmid pFX88. β subunit clusterin was also cloned into pCMV-Myc to obtain the expression plasmid pFX111.

HEK 293 cells were maintained in Dulbecco's Modified Eagle's Medium supplement with 12% fetal bovine serum. Ten μ g of each plasmid DNA were transfected into 5×10^5 HEK 293 cells using the calcium phosphate transfection kit (Invitrogen, Carlsbad, CA, USA). Cells were harvested 48 hours post-transfection in 500 μ l of lysis buffer, and used for co-immunoprecipitation with the mammalian HA-Tag IP/Co-IP kit (Pierce, Rockford, IL, USA). Proteins co-immunoprecipitated by anti-HA antibody were subjected to SDS-polyacrylamide gel electrophoresis on a 10% acrylamide gel, blotted on a nylon membrane and detected with mouse monoclonal anti-Myc antibody (Clontech) according to Amersham ECL Plus Western Blotting Systems (Piscataway, NJ, USA).

Circular dichroism analysis

CD spectra in the far-UV region were recorded between 200 nm and 300 nm on a Jasco J-810 Spectropolarimeter (JASCO, Japan) at 25 ± 0.2 °C in a rectangular quartz cuvette with 2 mm path length. The bandwidth was 1.0 nm with a resolution of 0.2 nm and a scan speed of 100 nm/min. CD spectra were accumulated from ten measurements. The ellipticity of CD spectra is expressed in millidegrees.

To provide comparable CD data, the concentrations of the protein samples were set as follows: the reference human PrPc sample (Prionics, Switzerland) comprised of 100 μ L of 8 μ M PrPc stock solution in phosphate buffered saline pH 7.4 (PBS) (Quality Biological, Inc., USA) and 200 μ L of PBS buffer. The reference human clusterin sample (ProSpec, Israel) comprised of 100 μ L of 8 μ M clusterin stock solution (ProSpec, Israel) and 200 μ L of PBS. The mixed PrPc/clusterin sample was obtained by adding of 100 μ L of PrPc stock solution and 100 μ L of clusterin stock solution to 100 μ L of PBS. Human serum albumin (ALB) essentially fatty acid free (Sigma Chemical Co., USA) was used in the same experimental conditions and comparable concentration as a negative control to illustrate a “no-binding” scenario. CD spectrum of PBS (background) was subtracted from each protein CD spectra.

RESULTS

Yeast two-hybrid screen

The human prion core region PrP²³⁻²³¹, without an N-terminal signal peptide and the C-terminal hydrophobic peptide, was cloned into the GAL4 DNA-binding domain bait vector pGBKT7 to create the bait plasmid pFX2. To isolate the putative prion interacting proteins, pFX2 was transformed into yeast AH109 and the resulting yeast AH109 [pFX2] was mated with a pretransformed human adult brain cDNA yeast library fused to the GAL4 activation domain (AD). The mating mixture was grown on selection SD medium in the absence of four nutrients (Leu, Trp, Ade, and His). Prior to performing the Y2H assay, the bait plasmid pFX2 was not found to spontaneously activate the reporter genes HIS3 and ADE2. After five days of growing on the SD selection medium, 2302 clones were obtained from 2.4×10^8 screened human adult brain cDNA clones. Of these, 250 clones were screened further on X-a-Gal medium and 249 were found to be positive. DNA from 110 positive clones were amplified with PCR and sequenced. One of these positive clones (Y2H3109) was found to encode the C-terminal region amino acids 215-449 of clusterin gene in the correct reading frame. One of the plasmids encoding clusterin in the yeast clone Y2H3109 was extracted and designed as pFX48. The pFX48 was reintroduced into yeast Y187 to obtain yeast Y187 [pFX48], and mated with prion bait yeast AH109 [pFX2]. The resulting yeast was retested on the SD selection medium, and positive clones were grown. This re-cloning further confirms the interaction between the PrPc and clusterin in yeast.

Clusterin α subunit but not β subunit binds to prion

Plasmid pFX48, isolated from the original yeast two hybrid screening, contained the entire a subunit of clusterin and additional 13 amino acids (clusterin 215-227) that encode a segment of the β subunit of clusterin. To determine whether the a subunit of clusterin can independently interact with the PrPc, the a subunit region was amplified, cloned into a pray pGADT7 vector to obtain pFX90, and introduced into yeast Y187. The resulting yeast Y187 [pFX90] was mated with prion bait yeast AH109 [pFX2], and streaked on SD selection medium. Positive clones were grown up on the SD selection medium. This result demonstrates the interaction between the PrPc and the a subunit of clusterin in yeast. In contrast, amplification of the clusterin β subunit fragment with PCR, cloning it into pGADT7 to obtain pFX77 and testing it in the Y2H assay resulted in no interaction between the PrPc and β subunit of clusterin in yeast.

The a helix region of clusterin a subunit binds to prion

To determine the smallest region of clusterin that interacts with the PrPc, several plasmids were constructed with clusterin sections of various sizes and tested with the Y2H assay.

The plasmid pFX48 containing the clusterin 215-449 was used to delete coding regions from N-terminal side to obtain the clusterin²⁷³⁻⁴⁴⁹ plasmid pFX64 or clusterin³⁰⁸⁻⁴⁴⁹ plasmid pFX73. Plasmid pFX64 and pFX73 were introduced into yeast Y187 respectively and mated with prion pray yeast AH109 [pFX2]; the resulting yeast was shown to be able to grow on SD

selection medium. These results indicate that both the clusterin C-terminal 273-449 region and an even shorter region 308-449 are able to interact with PrPc.

When the software PredictProtein[20] was used for clusterin sequence analysis, it revealed two a helix regions that are encoded around 320-374 and 428-447 respectively. To test whether the a helix region is essential for the interaction between clusterin and the PrPc, both clusterin 320-381 and clusterin 375-449 regions were amplified with PCR and cloned into pGADT7 to obtain the plasmid pFX97 and pFX81, respectively. Plasmid pFX97 and pFX81 were introduced into yeast Y187 and mated with prion pray yeast AH109 [pFX2]; the resulting yeast from mating with pFX97 but not pFX81 was capable of growing after four days on the SD selection medium. These observations indicate that the 62 amino acids of the clusterin a helix section 320-381 is able to interact with PrPc while a separate C-terminal a helix region 428-447 is not. Most likely there are additional factors besides the helix structure that facilitate the interaction with the PrPc.

Previously, the C-terminal coiled-coil domain of the nuclear form of clusterin was found to be essential for binding to Ku70 protein[21]. We amplified the coiled-coil domain region of clusterin³¹⁴⁻³⁴⁹ and cloned into pGADT7 to obtain the plasmid pFX92. Upon testing with prion AH109 [pFX2] by the Y2H assay, it did not bind to the PrPc. This result suggests that a different mechanism besides the coiled-coiled domain is involved in the interaction of clusterin with PrPc.

The clusterin a helix section 320-381 (pFX97) is able to interact with PrPc, but not through the coiled-coil domain region of this a helix section 314-349 (pFX92). To test whether the remaining region can bind to PrPc, clusterin 349-381 was amplified, cloned into pGADT7 to obtain the plasmid pFX110 and tested with prion AH109 [pFX2] by Y2H assay. Clusterin 349-381 was not able to bind to the PrPc. This demonstrated that the 62 amino acids of the clusterin a helix section 320-381 is the shortest region that is able to interact with PrPc.

Prion N-terminal region PrP²³⁻⁹⁵ and C-terminal region PrP⁹⁵⁻²³¹ binds to clusterin

Prion plasmid pFX2 which contains the mature prion coding region 23-231 was used to identify the clusterin as a prion ligand in the original Y2H library screening. To further characterize the prion region responsible for the clusterin binding, the N-terminal region PrP²³⁻⁹⁵ plasmid pFX33 and C-terminal region PrP⁹⁵⁻²³¹ plasmid pFX70 were constructed from pFX2, introduced into yeast AH109, mated with yeast Y817[pFX90] which contains the clusterin a subunit, and tested on SD selection medium. Both of AH109 [pFX33] and AH109 [pFX70] mated with Y187 [pFX90] were able to grow up on SD selection medium after three days (Fig. 1). Control AH109 [pGBKT7] mated with Y187 [pFX90] was not able to grow on SD-His-Leu-Trp-Ade selection medium, but was able to grow on SD-Leu-Trp medium. These data suggests that either the N-terminal region PrP²³⁻⁹⁵ or the C-terminal region PrP⁹⁵⁻²³¹ is able to interact with clusterin. A similar interaction of chaperone protein Hsp60 and PrPc was reported in a Syrian golden hamster

[8]. However, only the region represented by amino acids 180 to 210, but not the N-terminal region of Syrian golden hamster PrPc was involved in the binding to Hsp60. This suggests that there is a different mechanism involved in the chaperone protein clusterin and Hsp60 binding of human PrPc.

PrPc interacts with clusterin in mammalian cells

To examine whether PrPc can interact with clusterin *in vivo*, HA- PrP²³⁻²³¹ plasmid pFX43 and clusterin α subunit c-Myc-clusterin²⁷³⁻⁴⁴⁹ plasmid pFX 88 were cotransfected into HEK293 cells, grown and finally lysed. The cell extracts were co-immunoprecipitated with an anti-HA antibody and the immunoprecipitates were separated on Western blots. Myc-tagged clusterin co-immunoprecipitated with HA-tagged PrPc and was detected by anti-Myc antibody in Western blots (Fig. 2, Lane 5). No signal was detectable in cells transfected with Myc-tagged clusterin alone (Fig. 2, Lane 4) or HA-tagged prion alone (Fig. 2, Lane 3). Similar co-immunoprecipitation was performed for clusterin β subunit and PrPc but no signal for the clusterin β subunit was detectable (Fig. 2, Lane 2). Our data thus show that PrPc can interact with the clusterin α subunit but not the β subunit, in yeast and in mammalian cells. This result is consistent with previous studies in mammalian system in which clusterin was co-purified with PrPc from ram epididymal fluid[17] and was cross-linked with PrPc in a living mouse brain[18].

CD characterization of clusterin interactions with PrPc *in vitro*

The in vitro interactions between clusterin and PrPc were evaluated by far-UV CD spectrometry. CD spectroscopy provides a non-invasive spectral analysis which is particularly sensitive to protein conformational changes and thus, may provide a deeper insight into protein-protein interactions. As a negative control we utilized mixed samples of human albumin (ALB) with both clusterin and PrPc (experimental data for ALB and clusterin are shown in Fig.3A) that illustrate a “no-binding” case for which CD intensity of the mixed sample is in an excellent agreement with a mathematical sum of the CD intensities of each individual protein in the mixture. Fig. 3B shows far-UV CD spectra of clusterin and PrPc alone in aqueous solution (pH 7.4) with characteristic bands at 208 nm and 220 nm typical for α -helical structure. Similarly to the results shown in Fig. 3A, the absence of interactions between clusterin and PrPc should be reflected also by an additive type CD spectrum of the mixed sample. The thin grey trace on Fig. 3B represents a superposition of the CD spectra of PrPc and clusterin (shown in blue and purple) that was projected as a mathematical sum of both spectra and which would be evident if the proteins do not interact but co-exist independently in the same solution. However, the experimental CD spectrum of the mixed sample clusterin+PrPc was substantially different. Two kinds of significant CD spectral changes were observed after the PrPc was mixed with clusterin. First, the CD intensity of the PrPc+clusterin mixed sample dropped below the intensities of each individual spectra (blue and purple curves) and it was further contracting over time during the next 35 min. For clarity, only 60 min spectrum is shown in Fig. 3B (a thick red curve). This observation reflects significant conformational changes that proceeded over time upon protein-

protein interactions until a certain conformational equilibrium was achieved. Second, the change of the shape of PrPc+clusterin CD spectrum by flattening of 208 nm band clearly reflects the alterations in protein helicity upon protein-protein interactions, thus suggesting that the clusterin and PrPc do interact *in vitro*.

DISCUSSION

Previously, several groups have used the Y2H assay to screen prion binding proteins in yeast cotransformed with cDNA library and PrPc DNA. In this study, we employed a new Y2H assay in which a pretransformed cDNA library and the prion pray plasmid were introduced by mating instead of cotransformation. Our data demonstrates an interaction between prion and clusterin proteins using the Y2H assay and through a co-immunoprecipitation in transfected cells. In addition, CD spectrum analysis of the *in vitro* interaction between PrPc and clusterin provides clear evidence of conformational changes when PrPc and clusterin are mixed together.

Clusterin is a widely expressed glycoprotein, and its major secretory form is generated from cleavage of the full length protein at peptide bond Arg²²⁷-Ser²²⁸ to yield α and β polypeptides that are linked by five disulphide bonds to form the heterodimer. We found that the PrPc interacts only with the α but not with the β polypeptides. Furthermore, clusterin also exists as a nuclear form from alternative splicing[21], but this form is unlikely to interact with PrPc based on the subcellular distribution of the PrPc. Most of the PrPc are localized on the cell

membranes, where they are attached to the lipid bilayer via a C-terminal GPI anchor, although minute amounts of PrPc may exist as two transmembrane variants (^{Ntm}PrP and ^{Ctm}PrP)[22, 23].

Our findings indicate that specific regions of clusterin interact with the PrPc and are consistent with the following previous evidence pointing to clusterin as one of PrPc ligands. First, clusterin was found to prevent the aggregation of prion fragment 106-126 in vitro[24]. Second, clusterin was co-purified with soluble PrPc from ram epididymal fluid[17]. Third, clusterin was found residing in the molecular microenvironment of the PrPc in living mouse brain through time-controlled transcardiac perfusion cross-linking[18]. Fourth, clusterin has been shown to be associated with PrPc in TSE diseases. In the CNS and PNS of infected animals and humans, clusterin was co-localized with the extracellular plaque-type prion deposits, and its expression was increased in association with abnormal prion deposits[25-29]. Finally, clusterin knockout mice inoculated with BSE agent had an increased incubation time prior to symptom development in comparison with wild-type mice[19] which suggest that the pathophysiologic mechanism of this disease involves clusterin in some manner.

Clusterin is implicated in various cell functions that are involved in apoptosis, cell cycle control, DNA repair, lipid transportation, cell adhesion, and in diseases such as cancer, Alzheimer's disease and other neurodegenerative diseases[30-33]. Clusterin has been proposed to be a secreted mammalian chaperone [34]. Consistent with its putative extracellular chaperone function, clusterin prevents precipitation of denatured proteins in vitro[35] and mediates the

transport of the Alzheimer's disease A β peptide from the blood to the brain across the blood-brain barrier through the clusterin receptor[36]. Interestingly, there is 25% similarity between residues of 286-343 of clusterin and aB-crystallins [34]. aB-crystalline[9] and other chaperone proteins, Hsp60[8] and Rdj2[10], have been shown to directly interact with PrPc. In fact, aB-crystalline was identified as prion binding protein in the 110 sequenced clones obtained in our yeast two-hybrid screen. Both prion diseases and Alzheimer's disease underlying mechanisms involve a conformational change of normally expressed proteins without an alteration in their amino acid sequence[37, 38]. The involvement of clusterin in both of these neurodegenerative diseases raises the possibility of a common pathophysiologic mechanisms.

Currently, there is no treatment for TSE diseases. One rational approach to therapy is to develop a method(s) to specifically eliminate the PrPc or its ligands. Inhibition of PrP^{sc} formation in chronically prion-infected cells has been achieved by lentiviral gene transfer of the prion gene containing dominant negative mutations[39]. In addition, clusterin knockout mice inoculated with BSE agent had increased incubation time in comparison with wild-type mice[19]. These observations suggest that therapeutic targeting of the PrPc or its ligands, such as clusterin, could prevent or delay the onset of prion diseases. Phase II studies for clusterin as a therapeutic target by antisense clusterin oligodeoxynucleotides in malignancies are underway[40-42]. It is possible that the clinical modulation of clusterin may also be an effective therapeutic target for human prion diseases.

In conclusion, we confirmed that clusterin is a ligand for human PrP^c. Elucidation of the molecular mechanism of the interaction between prion and clusterin proteins will help to further understand the pathogenesis of TSE diseases and potentially provide future therapeutic approaches.

ACCEPTED MANUSCRIPT

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Table 1: Primers

Primer	Sequence	Restriction Site	Gene (aa position)
FXF3	TGAAGATACCCACCAAACC	--	pGADT7
FXR3	GCACGATGCACAGTTGAAGT	--	pGADT7
FXF1	AAATCGGAATTC <u>AAGAAGCGCCCGAAGCCTGG</u>	<i>EcoRI</i>	PRNP (23)
FXR1	TTTAGC <u>GGATCC</u> GCTCGATCCTCTCTGGTAATAGG	<i>BamHI</i>	PRNP (23)
FX21F	AAGGATCCATGAATTCGACCAGACGGTCTCAGACAATG	<i>BamHI</i>	CLU (23)
FX21R	AACTCGAGTCGACCGGACGATGCGGGACTTGGGA	<i>XhoI</i>	CLU (227)
FX22F	ATAGGATCCatGAATTCATACGAGAAGGCGAC	<i>BamHI</i>	CLU (273)
FX22R	ATACTCGAGTCGACTCACTCCTCCCGGTGCTTTT	<i>XhoI</i>	CLU (449)
FX23F	ATAGGATCCTCACGCAAGGCGAAGACCAG	<i>BamHI</i>	CLU (375)
FX25F	ATAGGATCCATAGCTTGATGCCCTTCTCTCCG	<i>BamHI</i>	CLU (228)
FX27F	ATAGGATCCATCAGGCTAAGCTGCGGCGGGAGC	<i>BamHI</i>	CLU (320)
FX28F	ATAAGGATCCATACCAACAACCCCTCCAGGC	<i>BamHI</i>	CLU (314)
FX28R	ATAACTCGAGTCGACTGGTAGGACTTTAGCAGCTC	<i>XhoI</i>	CLU (349)
FX29R	ATAACTCGAGCTGGTCTTCGCCTTGCGTGAGG	<i>XhoI</i>	CLU (381)
FX36F	ATAGGATCCAGTGGAAGATGCTCAACACC	<i>BamHI</i>	CLU (349)

Table 2: Plasmids

Plasmid	Gene (aa region)	Protein region	Vector	Plasmid generated from
pFX1	PRNP (aa 23-231)		pGEM3Z	
pFX2	PRNP (aa 23-231)		pGBKT7	
pFX33	PRNP (aa 23-95)	PrPc N-terminal	pGBKT7	
pFX43	PRNP (aa 23-231)		pCMV-HA	
pFX70	PRNP (aa 95-231)	PrPc C-terminal	pGBKT7	
pFX48	CLU (aa 215-449)	Clusterin a subunit	pGADT7-Rec	Y2H
pFX64	CLU (aa 273-449)	Clusterin a subunit	pGADT7-Rec	pFX48
pFX73	CLU (aa 308-449)	Clusterin a subunit	pGADT7-Rec	pFX48
pFX75	CLU (aa 23-227)	Clusterin β subunit	pSC-A	human clusterin plasmid
pFX77	CLU (aa 23-227)	Clusterin β subunit	pGADT7	human clusterin plasmid
pFX79	CLU (aa 375-449)	Clusterin a subunit	pSC-A	pFX64
pFX81	CLU (aa 375-449)	Clusterin a subunit a Helix	pGADT7	pFX64
pFX88	CLU (aa 273-449)	Clusterin a subunit	pCMV-Myc	human clusterin plasmid
pFX89	CLU (aa 228-449)	Clusterin a subunit	pSC-A	human clusterin plasmid
pFX90	CLU (aa 228-449)	Clusterin a subunit	pGADT7	human clusterin plasmid
pFX92	CLU (aa 314-349)	Clusterin a subunit coiled-coil leucine zipper-like	pGADT7	human clusterin plasmid
pFX95	CLU (aa 320-381)	Clusterin a subunit a Helix	pSC-A	human clusterin plasmid
pFX97	CLU (aa 320-381)	Clusterin a subunit a Helix	pGADT7	human clusterin plasmid
pFX110	CLU (aa 349-381)	Clusterin a subunit a Helix	pGADT7	human clusterin plasmid
pFX111	CLU (aa 23-227)	Clusterin β subunit	pCMV-Myc	human clusterin plasmid





