Identification of proteins co-purifying with scrapie infectivity

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ARTICLE DATA

PrPC, the cellular isoform of prion protein, is widely expressed in most tissues. Despite its involvement in several bioprocesses it still has no apparent physiological role. During propagation of Transmissible Spongiform Encephalopathies, PrPC is converted to the pathological isoform, PrPSc, in a process believed to be mediated by unknown host factors. PrPSc has altered biochemical properties and forms amyloid aggregates that display infectious characteristics. PrPSc is also the major component in biochemically enriched infectious samples. Other molecules co-purify with it, but the protein content of these aggregates remains unknown. The goal of this project was to identify other host molecules with high affinity for PrPSc. Here, we present the identification of protein molecules that co-purify with PrPSc isolated from naturally scrapie-infected ovine brain tissue. The infectious preparations were analyzed by two-dimensional gel electrophoresis and unknown proteins were identified by LC-MS/MS. These proteins may prove to be strategic targets for prevention and therapy of prion diseases.

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1. Introduction

Transmissible Spongiform Encephalopathies (TSE) are a group of fatal neurodegenerative diseases characterized by the formation of amyloid aggregates and vacuolation of brain tissue. They are caused by the conversion of a physiological cellular protein, PrPC, to an insoluble and infectious isoform, termed PrPSc [1]. The conversion mechanism remains undefined, but it is proposed to be mediated by as yet unknown host-encoded factors [2]. PrPSc represents the main component of the infectious agent and has the ability to accumulate in extracellular fibrillar aggregates.

PrPC, the physiological prion protein is attached to the outer cell membrane via a glycosylphosphatidylinositol (GPI) anchor. Even though it is expressed in most tissues [3,4], still it has no definite biological function. Several attempts have been made to identify molecules that interact with PrPC in order to understand its function and the mechanism of its conversion to PrPSc [5–8]. However, little is known concerning proteins that co-purify with TSE aggregates.

In this study we tried to identify proteins in highly-enriched infectious preparations, using a proteomic approach. Proteins associated with the pathological isoform of PrP, originating from scrapie-infected brain tissue, were identified by the combination of two-dimensional gel electrophoresis (2-DE)
and mass spectrometry. The results showed that amyloid aggregates contain components of the extracellular matrix (ECM) and proteins related to it. The results presented here suggest that these molecules correlate with prion infectivity and may participate in the pathogenesis of TSE.

2. Materials and methods

2.1. PrPSc enrichment

Ovine cortex from normal or scrapie infected sheep were kindly provided by Dr. P. Toumazos (Veterinary Services Laboratory, Cyprus). The tissues were used for PrPSc enrichment, as previously described [9]. In brief, 500 mg brain tissue was homogenized in nine volumes of 10% w/v Sarkosyl, 25 mM Tris–Cl pH 7.6 with the addition of 0.5 mM PMSF. Homogenization was performed using a polytron homogenizer (Kinematica, Switzerland) at setting 3, for 3×15 s at room temperature. The homogenates were centrifuged at 25×103 g for 30 min at 20 °C. The ultracentrifugation pellet was resuspended in Buffer A (0.05% w/v Sarkosyl, 25 mM Tris–Cl pH 7.6) overnight at room temperature. The resuspension was centrifuged at 20×103 g for 30 min and was washed twice in Buffer B (0.05% Sarkosyl, 25 mM Tris–Cl pH 7.6). The final pellet was treated with or without Proteinase K (PK) and analyzed by electrophoresis.

2.2. Proteinase K treatment

The final pellet was resuspended in 100 µl Buffer B and treated with 30 µg/ml PK for 1 h at 37 °C with constant shaking (800 rpm/min). PMSF was added (0.5 mM), followed by centrifugation at 20×103 g for 30 min. The pellet was resuspended in 500 µl Buffer A for 2 h at room temperature and then centrifuged at 20×103 g for 30 min. The final pellet was washed twice in Buffer B before analyzing in two-dimensional electrophoresis.

2.3. Two-dimensional gel electrophoresis

The final pellet was resuspended in 6 M Urea, 2 M Thiourea, 4% w/v CHAPS, 60 mM DTT, 2% ampholytes pH 3–10, 0.1% w/v Bromophenol Blue and sonicated in a bath sonicator for 5 min. For isoelectric focusing (IEF) using immobilized pH gradient (IPG) strips in the first dimension, precast gels with a non-linear pH range of 3–10 (Zoom, Invitrogen) were reswollen for 16 h in 180 µl of the resuspended sample. IEF was carried out at room temperature for 20 min at 200 V, 15 min at 450 V, 15 min at 750 V and 105 min at 2000 V for, using a cooled electrophoresis unit (Zoom IPG runner, Invitrogen). For the second dimension, IPG strips were equilibrated for 15 min in 6 M Urea, 2 M Thiourea, 60 mM DTT Invitrogen LDS sample buffer, followed by equilibration for 15 min in 6 M Urea, 2 M Thiourea, 2.5% w/v Iodoacetamide Invitrogen LDS sample buffer. The strips were placed on 4–12% gradient NuPAGE Novex Bis–Tris–Gels and proteins were analyzed at 200 V for 1 h in MES buffer.

2.4. Silver protein staining

Silver protein staining was performed as previously described [10]. In brief, proteins were fixed in the gel with 50% v/v methanol, 5% v/v acetic acid for 1 h, followed by incubation with 50% v/v methanol for 15 min and dH2O for another 15 min. After fixation, the gels were immersed in 0.02% w/v sodium thiosulfate for 3 min and washed with dH2O for 2×1 min. The gels were then incubated with 0.15% w/v silver nitrate for 45 min. After 2×1 min washes in dH2O, proteins were visualized with 0.04% v/v formaldehyde, 2% w/v sodium bicarbonate. The reaction was quenched with 5% v/v acetic acid and the gels were stored in 10% v/v methanol, 1% v/v acetic acid.

2.5. Western blotting

Proteins were electrotransferred onto nitrocellulose (NC) membranes (Pall Life Sciences) at 100 V for 2 h. The membranes were blocked with 5% w/v BSA (Sigma-Aldrich) in PBST for 1 h at room temperature. Prion protein was detected with anti-PrP mAb 6H4 (Prionics, 1:3000 in PBST) overnight at 4 °C. The membrane was washed in PBST and incubated with rabbit anti-mouse alkaline-phosphatase conjugated secondary antibody (Pierce, 1:5000 in PBST), for 1 h at room temperature. After washes with PBST, blots were developed with Nitro Blue Tetrazolium/5-Bromo-4-Chloro-3-Indolyl Phosphate disodium salt (NBT/BCIP) (Sigma-Aldrich). For non-specific staining NC membranes were stained with Aurodye (Amersham Pharmacia) according to manufacturer’s instructions.

2.6. LC-MS/MS analysis and protein identification

Proteins co-purifying with PrPSc were analyzed in 2-DE and stained with silver solution. Prior to analysis the gel slices containing the desired spots, were subjected to a standard procedure during which proteins were reduced with 100 mM DTT for 30 min at 56 °C, alkylated with iodoacetamide in darkness for 45 min at room temperature and digested overnight with trypsin. The resulting peptides were eluted from the gel with 0.1% TFA and 2% ACN. Peptide mixtures were applied to an RP-18 pre-column (Waters) using 0.1% TFA in dH2O as mobile phase and then transferred to a nano-HPLC (NanoACQUITY UPLC, Waters) RP-18 column (75 µM i.d., Waters.) with an acetonitrile gradient (0%–30% AcN in 45 min) in the presence of 0.1% formic acid (flow rate 250 nl/min). The column outlet was directly coupled to the ESI (Finningan Nanospray, Thermo) ion source of the LTQ-FT (Thermo) mass spectrometer working in the regime of data dependent MS to MS/MS switch. A blank run ensuring lack of cross contamination from previous samples preceded each analysis. Source voltage and current were set to 1.6 kV and 100 µA, respectively. The capillary voltage was 42 V and tube lens 120 V. FTMS resolution was set to 12,500 and m/z values from range 300–2000 Th were acquired. Minimal signal required to trigger MS to MS/MS switch was set to 500,
activation Q was 0.250 and normalized collision energy was 35. The spectrometer was working in positive polarity mode and charge states accepted for sequencing were 2+, 3+ and 4+. Dynamic ion exclusion was enabled and exclusion time was set to 300 s.

After preprocessing of the raw data with Mascot Distiller software (version 2.1.1, Matrix Science), output lists of precursor and product ions were compared to National Center for Biotechnology non-redundant (NCBInr) database (containing 3695564 sequences; 1269795892 residues) using Mascot database search engine (version 2.1, Matrix Science). Search parameters included semi-trypsin enzyme specificity, one missed cleavage site, cysteine carbamidomethyl fixed modification and variable modifications including methionine oxidation and lysine carbamidomethylation. Protein mass and taxonomy were unrestricted, peptide mass tolerance was 200 ppm and the MS/MS tolerance was 0.8 Da. Proteins containing peptides with Mascot cut-off scores >50, indicating identity or extensive homology ($p<0.05$) of peptide, were considered positive identifications.

### 3. Results and discussion

In order to identify novel molecules that may participate in TSE pathogenesis, scrapie-infected ovine brain tissue was homogenized and enriched in the pathological isoform of prion protein, PrPSc. The final preparation, a pellet obtained after ultracentrifugation at $215 \times 10^3$ g for 2 h 30 min, contains almost 95% of the infectivity and displays unique infectious characteristic [9]. The presence of PrPSc was verified by western blotting (Fig. 1A). Additionally, the same preparation was stained with non-specific protein staining solution (Fig. 1B) which showed that these samples are highly enriched in the pathological isoform.

In order to identify the unknown proteins that, together with PrPSc, precipitated at $215 \times 10^3$ g, samples were digested with PK and analyzed in a 2-DE. The protein content of the sample (5 times higher than the amount used for 1-DE gel electrophoresis) was focused in non-linear pH 3–10 IPI strips before separation in a pre-cast gradient 4–12% NuPAGE gel. After 2-DE analysis, proteins were stained with mass-spectrometry compatible silver nitrate solution. Control experiments were performed in brain preparations originating from healthy individuals, in which a significantly lower amount and pattern of protein was detected (Supplementary Fig. 1). However, the pellets resulting from healthy and infected individuals have totally different biochemical features and are not comparable. As shown in Fig. 2, several proteins (including PrPSc) were detected in the infectious preparation after PK treatment. Experiments were performed three times and in each case the observed protein pattern was reproducible. As this pellet contains the major infectivity components, we were interested in identifying most of the molecules that participate in its formation and are good candidates to be part of the infectious agent. The molecules indicated by numbers were manually excised from the gel and analyzed by LC-MS/MS (Table 1). Proteins were mainly identified by similarities with homologous proteins from other species, as the ovine genome is not completely sequenced and many ovine proteins are not available in the database. The mass spectrometry score for each molecule was calculated based on the ion score of the peptides identified and attributed to the respective protein. It is important to note that the electrophoretic mobility of most spots was not consistent with the expected molecular weight of the identified proteins, as the sample was treated with PK before analysis, possibly resulting in partial fragmentation of the molecules. In three of the spots we identified various types of collagen (spots 1, 3 and 5). Additionally, we identified components of the desmosomes, such as desmoglein 1 (DSG1, spots 2 and 3) and plakoglobin (JUP, spot 2). The infectious pellet also contained ubiquitin (spot 9), versican V3, a splicing variant of a large chondroitin sulfate proteoglycan (spot 4) and brain link protein-1 (BRAL1), a protein which is directly related to versican (spot 9). In spots 6–8 we identified the alpha subunit of the calcium/calmodulin-dependent protein kinase 2 (CAMK2A, spot 8), the light and heavy chains of ferritin (FTL and FTH, spots 6 and 8, respectively) and prion protein, as
expected. The identification of more than one protein in some spots is possibly due to sample overlapping, especially in the lower molecular weight area of the gel.

The goal of this project was to identify proteins that co-purify with the infectious isoform of prion protein in PrPSc enriched brain preparations. Despite the fact that several ligands of the physiological prion protein, PrPC, have already been identified, the protein composition of TSE aggregates remains unknown. These aggregates display unique infectious characteristics, as they can effectively transmit the disease to healthy individuals. The results showed that the infectious preparation contains mostly proteins of the ECM, such as various types of collagen, proteoglycans (versican V3) and molecules related to them (BRAL1). Additionally, we identified components of the desmosomes (DSG1, JUP), ubiquitin, ferritin and CAMK2A.

To minimize non-specific protein isolation, we used very stringent conditions, washing the pellet extensively with buffers containing high concentration of salts (10 w/v NaCl). However, there is always a possibility that some of these molecules may not specifically associate with PrPSc, but coincidentally co-purify with it because of the experimental procedure we followed. The identification of glycoprotein binding molecules (versican V3 and BRAL1) or CAMK2A, which is involved in ER stress under neurodegenerative conditions [11] indicates the specificity of PrPSc co-purifying proteins. The detection of ubiquitin in prion aggregates provides another piece of evidence, as protein aggregates involved in most neurodegenerative diseases are highly ubiquitinated [12]. It also has to be mentioned that this fraction is one of the most extensively purified infectivity preparations that is reported in the TSE field. Thus, the presence of the identified proteins in this preparation makes them candidate molecules directly associated with prion infectivity.

ECM consists mainly of glycosylated proteins, such as collagen, proteoglycans (PGs) and hyaluronic acid. It is located in the space between cells and has multiple roles. It provides the cells with the essential mechanical support, as it is connected through the integrins to the cytoskeleton. The whole system requires the presence of the desmosomes and hemidesmosomes which allow intercellular connections and stabilize the cells to the ECM, respectively. ECM also regulates cellular communication and activity of the ion channels located in the cell membrane. The regulation mechanism requires the interaction of PGs with membrane glycoproteins, which activate intracellular phosphorylation systems, such as protein kinase II [13].

The PGs located in the ECM seem to be involved in Alzheimer’s disease, as they can act as seed cores for the formation of amyloids [14]. Additionally, heparan sulfate, the most abundant proteoglycan of ECM, accumulates in the amyloid plaques of Gerstmann–Straussler syndrome disease patients [15] and is directly related to prion infectivity [16]. It is therefore, possible that proteins such as versican V3 and

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<th>Table 1 – Proteins identified in PrPSc enriched pellets.</th>
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<td>Spot</td>
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<tr>
<td>1 COL6A1</td>
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<td>2 DSG1</td>
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<td>3 JUP</td>
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<td>4 COL1A2</td>
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<td>5 Versican V3</td>
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<td>9 BRAL1</td>
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<td>10 Ubiquitin</td>
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Table shows PrPSc associated proteins which were identified in each spot after LC-MS/MS analysis. It also indicates the protein score, the number of matching peptides used for protein identification, the sequence, mass, m/z and charge of each peptide.
BRAL1, which were identified in the infectious preparations, participate in the conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup> and the propagation of prions to the central nervous system.

Identification of ferritin in the infectious preparation is also quite interesting. Experimental data show that ferritin and PrP<sup>Sc</sup> form complexes and are co-transported through receptors to epithelial cells of the gastrointestinal tract [17]. Hypothetically, a similar mechanism could also exist for the transportation of the infectious isoform to the CNS and the crossing of the blood brain barrier.

In summary, the experimental data presented in this paper show that the infectious isoform of prion protein is directly related to ECM and proteins associated with it. These proteins may prove to be therapeutic targets for the prevention of prion diseases. Further experiments are needed in order to study in detail their potential participation in the conversion mechanism of PrP<sup>C</sup> to PrP<sup>Sc</sup> and pathogenesis of TSE.

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jprot.2009.01.025.

**REFERENCES**