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Immunoreactivity enhancement with chelators for increasing the detection sensitivity of human PrP^{Sc} by Western blotting

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Abstract

Prion diseases are neurodegenerative disorders affecting humans as Creutzfeldt–Jakob disease. The host-encoded prion protein (PrP^{C}) will be converted into a structurally altered isoform (PrP^{Sc}). PrP^{Sc} differ in sizes and glycoform patterns and can be identified using molecular typing with Western blotting. The electrophoretic mobility of PrP^{Sc} changes on treatment with metal ions or chelators prior to digestion with proteases. The effects of chelators applied to PrP^{Sc} after protease digestion had not been examined in detail, we investigated these effects in this study. Application of EDTA, NTA and DTPA, and to a lesser extent EGTA, significantly enhanced PrP^{Sc} signals in immunoblots. PrP^{Sc} intensities increased two- to three-fold compared with untreated PrP^{Sc}. Since the immunoblot method is highly specific, sensitivity is the limiting factor. Enhancing sensitivity might be important in the determination of PrP^{Sc} at levels close to or just below the limits of detection. It is to be expected that application of chelators to digested protein samples will increase the sensitivity of PrP^{Sc} detection using the Western blot technique.

Keywords: Prion protein; Signal enhancement; Chelator; Protease

1. Introduction

Prion diseases are fatal neurodegenerative disorders that include bovine spongiform encephalopathy (BSE) in cattle and scrapie in sheep and goats. Human forms of the diseases include sporadic Creutzfeldt–Jakob disease (CJD), familiar and genetic linked CJD as well as infectious forms such as iatrogenic CJD and the variant CJD (vCJD) that has been linked to BSE (Collinge et al., 1996; Collinge, 2005). The disease is characterized by rapidly progressing dementia, ataxia and neurologic dysfunctions leading to death. The disease is characterized by the accumulation of an abnormal prion protein (PrP^{Sc}) formed by conversion of a cellular prion protein (PrP^{C}) in a post-translational process. Whereas, PrP^{C} contains mainly α-helix type structures, PrP^{Sc} has a β-sheet structure, which is responsible for a change in the biochemical properties of the molecule (Pan et al., 1993). In contrast to PrP^{C}, PrP^{Sc} is infectious, detergent insoluble, aggregates *in vitro* to form scrapie associated fibrils (SAFs) and is partially resistant to proteases. When incubated with proteinase K (PK), PrP^{C} is completely digested, whereas, PrP^{Sc} is truncated to a PK-resistant fragment. PK-treatment of prion-infected brain homogenates results in molecular degradation of the di-, mono- and non-glycosylated PrP^{Sc} isoforms by 6–8 kDa. Immunological assays for the detection of PrP^{Sc} are widely used in research and routine diagnosis. PrP^{Sc} isolates and strains are currently characterized on the basis of molecular properties of associated PrP^{Sc} as observed using Western blot techniques by differences in the molecular masses and the ratios of the signal intensities of the di-, mono- and non-glycosylated isoforms (Groschup et al., 2000; Gambetti et al., 2003; Hill et al., 2003). The finding of multifaceted prions suggests that other types of prions exist, and this supported by data obtained in a patient with multiple prion proteins (Polymenidou et al., 2005; Yull et al., 2006). At least five distinct subtypes of sCJD, differing in molecular mass and glycoform, have been reported (Parchi et al., 1999). One of these is the variant of CJD (vCJD) with a profile similar to that in cattle BSE. At least two types of CJD, differing in PrP^{Sc} migration, have been described (Gambetti et al., 2003; Hill et al., 2003). Type 1 is a non-glycosylated protein fragment migrating at

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21 kDa and type 2 is a 19 kDa fragment. These differing electrophoretic mobility characteristics are due differences in PK cleavage site (amino acid position 82 in type 1 and amino acid 97 in type 2).

Metal ions, e.g. copper, which attach to the amino terminal octapeptide region, have an effect on PK cleavage and also cause a change in the mobility of PrP^{Sc} fragments in the presence of metal chelators such as EDTA (Wadsworth et al., 1999). EDTA is a broad specifity chelator with high affinity for divalent cations such as magnesium but other chelators such as EGTA are more selective than EDTA and have a high affinity for copper ions. Differences in mobility can also arise from variations in pH (Zanusso et al., 2001; Notari et al., 2004) because the stoichiometry of copper binding is pH-dependent (Miura et al., 1999; Kramer et al., 2001). In these investigations, the effect of metal chelators has been determined prior to PK treatment. In the study reported here, however, the impact of metal chelators on the characteristics of PrP^{Sc} was determined after protease treatment. It could be shown that application of metal chelators leads to a marked enhancement of PrP^{Sc} signals providing the basis for a new approach to molecular typing.

2. Materials and methods

2.1. Antibodies and reagents

Monoclonal anti-PrP antibodies (mabs) were used for sensitive and specific prion detection. Mab SAF34, which binds to the entire octapeptide region of prion proteins (amino acids 59–89) and was produced by immunizing knock-out mice with proteinase K-treated, formic acid-denatured hamster scrapie associated fibrils (263K) (Demart et al., 1999). Mab 3F4 recognizes the epitope of amino acids 109–112 (Signet, USA) (Chen et al., 1995) and antibody P4 binds to residues 93–99 of ovine PrP (r-Biopharm, Germany) (Thuring et al., 2004).

The chelators ethylene-dinitrilo-tetra-acetic acid (EDTA) and ethylene-bis(oxyethylene-trinitrilo)-tetra-acetate (EGTA) (Sigma, Germany) were prepared as 500 mM stock solutions in water and adjusted to pH 8 using NaOH. Nitrilotriacetic acid (NTP) and diethylene-triamine-penta-acetic acid (DTPA) (Sigma, Germany) were prepared as 450 and 480 mM stock solutions in 50% (v/v) ethanol/water, respectively. Chelator suspensions at the concentrations shown were added to aliquots of homogenates after protease treatment.

2.2. Brain tissues and precipitation

Brain tissue containing PrP^{Sc} was obtained from a patient who had died from Creutzfeldt–Jakob disease (sporadic case) and from cattle diseased with BSE. Protein samples (10%, w/v) were prepared by homogenizing in nine volumes of lysis buffer (320 mM sucrose, 0.5% (w/v) igepal and 0.5% (w/v) sodium dodecyl-sulphate in Tris-buffered saline (pH 7.4; Sigma, Germany) in a glass homogenizer followed by intensive ultrasonification. After centrifugation at 900 × *g* for 5 min supernatants were stored at −20 °C until used.

2.3. Proteases treatment

Proteolytic assays were performed using proteinase K (PK), bromelain and chymotrypsin purchased as purified and crystalline material (Sigma, Germany). For digestion protein samples were incubated with chymotrypsin at room temperature and with bromelain and PK at 37 °C each for 60 min with mild rocking. The relative activity of individual proteases required to produce hydrolysis of PrP^{C} isolated in brain homogenates was calculated. The concentrations of the proteases used in the assays were 200 μg/ml for PK, 1 mg/ml for chymotrypsin and either 50 or 200 μg/ml in the case of bromelain as indicated. Hydrolysis was terminated by addition of a stop mixture of protease inhibitors containing phenylmethylsulfonyl fluoride (PMSF), tosylphenylalanylchloromethyl ketone (TPCK) (VWR, Germany) and Pefabloc (Roche, Germany) such that the final concentrations of each inhibitor were in the range 3–5 mM.

2.4. PTA precipitation

Sodium phosphotungstic acid precipitation was carried out as described with slight modifications (Gretzschel et al., 2005). Aliquots of 100 μl each of 10% (w/v) brain homogenates were treated with proteases as indicated and then adjusted to a final concentration of 50 U/ml with benzonase (Sigma, Germany) and 1 mM magnesium chloride. After incubation at 37 °C for 30 min, 100 μl TBS containing 4% (w/v) sarkosyl were added followed by further incubation at 37 °C for 30 min. Samples were adjusted to a final concentration of 0.3% (w/v) PTA with a stock solution consisting of 4% (w/v) sodium phosphostungstic acid (PTA) and 34 mM magnesium chloride and incubated at 37 °C for 60 min. Proteins were obtained by centrifuging at 14,000 × *g* for 30 min at room temperature and the pellets resuspended in SDS-loading buffer.

2.5. Immunoblot analysis

Samples were boiled for 5 min prior to separation of the proteins using sodium dodecyl-sulphate polyacrylamide (13%) gel electrophoresis (SDS-PAGE) in a mini slab gel apparatus (Bio-Rad, Germany). After electro-blotting onto polyvinylidene diflouride (PVDF) membranes (Roth, Germany) using a semidry blotting system (Roth, Germany), membranes were blocked in TBS containing 0.1% Tween 20 (TBST) and 1% (w/v) non-fat dry milk powder for 60 min. For specific binding of the antibodies to PrP proteins, membranes were incubated as indicated with the anti-PrP antibodies at room temperature overnight. Horseradish peroxidase-conjugated, affinity purified goat anti-mouse IgG (Dianova, Germany) served as secondary antibody. Protein signals were visualized using a chemiluminescence enhancement kit (Pierce, Germany). The signal intensities of the di-, mono- and non-glycosylated PrP isoforms, determined as total PrP signals with the Quantity one software (Bio-Rad, Germany), were used for quantitative analysis.

3. Results

3.1. Application of chelators increase human CJD signals in immunoblots

Prion proteins in a human CJD brain homogenate were analysed using immunoblotting with mab SAF34, which recognizes the 59–89 amino acid sequence of PrP. On treatment with proteases, pathological prion proteins (PrP^{Sc}) show a decrease in molecular mass of approximately 6–8 kDa. Because immunoblotting is a highly specific technique, detection is often limited by low sensitivity.

Application of the chelators EDTA, NTP and DTPA to protease treated samples markedly increased the signal intensities of the di-, mono- and non-glycosylated protein bands in human PrP^{Sc} immunoblots. The effect using EGTA was lower than those with the other two chelators when added at a final concentration of 20 mM (Fig. 1A). More over, the enhancement using chelators enabled the signal to be clearly observed even when the amount of PrP^{Sc} protein loaded onto the gels was at the detection limit in the absence of chelator treatment. This effect is due to the application of the chelator and not to differences in proteolysis since digestion of the protein samples and the addition of an equal volume of the chelators were carried out in the same tube. It is very unlikely that this effect is related to the PK treatment since chelators were introduced after a complete blocking of protease activity.