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Novel assay with fluorescence-labelled PrP peptides for differentiating L-type atypical and classical BSEs, and scrapie

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ABSTRACT

Characteristic differences of prions may account for the conformational diversity of the pathogenic isoform of prion protein (PrP^{Sc}). Here, we applied a protein detection procedure by using fluorescent-labelled peptides for detecting PrP^{Sc}. Five prion protein (PrP) related peptides were found to change significantly their fluorescent intensities with prion-affected animal samples. Their reactivity was different among atypical L-BSE, classical BSE and scrapie. The pull-down assay revealed that they precipitated PrP^{Sc} specifically. These findings suggest that fluorescent intensity changes depend on peptide–PrP^{Sc} binding. This novel approach may distinguish the fine structural differences in PrP^{Sc}, which were not detected by the pull-down assay.

Structured summary of protein interactions:
PrP-peptides HPP01, 02, 03, 06, 11 physically interact with **PrP^{Sc} of L-type atypical and classical BSEs, and scrapie** by pull down

1. Introduction

Prion diseases, also called transmissible spongiform encephalopathies (TSEs), are fatal neurodegenerative disorders, including bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goats, chronic wasting disease (CWD) in cervids, and Creutzfeldt–Jakob disease (CJD) in humans [1]. The pathogenic isoform of prion protein (PrP^{Sc}) is generated by post-translational modification of the cellular prion protein PrP^{C}, and is considered a major component of the infectious agent, prion [1]. Although PrP^{C} and PrP^{Sc} have identical amino acid sequence, their secondary and/or tertiary structures differ. Furthermore, prions consist of multiple types that are classified by incubation periods and lesion profiles in inbred mice [2]. Some prions may also differ in their PrP^{Sc} properties, e.g. different electrophoretic mobilities [3], relative glycoform ratios [4], N-terminal ends of proteinase K (PK) digested PrP^{Sc} [5], immunoreactivities against conformational-specific antibodies [6,7], and stabilities against chaotropes [8]. These differences suggest that prion identity is encoded by the conformation of PrP^{Sc}, but little research has been done to confirm this.

To differentiate among PrP^{Sc} conformers, it is necessary to develop a new strategy involving the use of probes that specifically bind to PrP^{Sc}. PrP^{C} fused with immunoglobulin [9] and PrP-grafted antibodies [10–12] specifically bind to PrP^{Sc}. Furthermore, several PrP synthetic peptides were shown to bind to PrP^{Sc} [13]. These results may indicate that PrP^{C} and PrP peptides bind to PrP^{Sc}, and therefore, could be developed as novel probes to classify PrP^{Sc} conformers.

Luminescent conjugated polymers (LCPs) could distinguish prion strains by their fluorescence spectra [14]. Non-covalent binding to proteins constrains the rotational freedom of LCPs, and thus alters their spectral properties in a conformation-dependent manner [15,16]. Monitoring the changes in fluorescence could be a sensitive method for detecting conformational variability in prion strains. A similar procedure has been reported for detecting peptide–protein binding by using fluorescence-labelled structurally designed peptides [17–20]. In this assay, the altered intensity of fluorescence represented the peptide–protein interactions.

Applying this technique for the analysis of prion differentiation, we now report that different peptides reacted with PrP^{Sc} derived from scrapie and those derived from the two types of BSE. Changes in fluorescence may represent a fine peptide–PrP^{Sc} interaction, which was not detected by the protein-pull down assay.

Abbreviations: PrP, prion protein; PrP^{C}, cellular prion protein; PrP^{Sc}, disease-associated prion protein; BSE, bovine spongiform encephalopathy; C-BSE, classical BSE; L-BSE, L-type atypical BSE; PK, proteinase K; FI, fluorescence index

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2. Materials and methods

2.1. Peptides

We synthesised a set of PrP-related peptides (HPP01–25) that were composed of 15 amino acids, shifted by 10 amino acids, and covered the entire human PrP sequence (GenBank Acc. No. AAH12844). Since the peptides corresponding to the N-terminal signal sequence (HPP01 and 02) or the C-terminal glycosylphosphatidylinositol (GPI) anchor signal (HPP24 and 25) were less soluble, an additional Lys-Lys-Arg (KKR) sequence was attached to their C-termini. The HPP06-s was generated as a randomly scrambled HPP06 peptide. Sequence details are shown in Table 1. The N-terminal end of the peptide was conjugated with an additional Gly residue as a spacer, along with either a fluorescent dye (TAMRA) for fluorescence-based detection or biotin for the pull-down assay. The C-terminal end of the peptide has an amide group. All peptides were synthesised by the solid-phase method using the Fmoc strategy [21].

2.2. Animals and prions

All animal experiments were reviewed by the committee dealing with Ethics in Animal Experiments at National Institute of Animal Health. The animals were sacrificed during the clinical stage of prion disease. The brains of mice (Slc:ICR) infected with the mouse scrapie strain (Chandler) [22] were used. The brainstems of sheep experimentally infected with scrapie [23] and cattle experimentally infected with classical BSE (C-BSE) [24] or L-type BSE (L-BSE) [25,26] were used. The brains of uninfected mice (Slc:ICR), and the brainstems of healthy sheep and cattle were used as negative controls.

2.3. Fluorescent peptide assay

Brain samples were homogenised in 4 volumes of 10 mM sodium phosphate buffer (pH 7.0) at 3000 rpm for 2 min by using a Multi-beads Shocker (Yasui-Kikai, Osaka, Japan) to produce a 20% (w/v) brain homogenate. Equal volumes of the brain homogenate and fluorescence-labelled peptide (2% (w/v) or 2 μM in 10 mM sodium phosphate buffer) were mixed in one well of Black Maxisorp Fluoronunc Cert. (ThermoFisher Scientific, Roskilde, Denmark), and incubated for 30 min at room temperature. Fluorescence was measured using an ARVO 1420 Multilabel Counter (Wallac-Perkin Elmer, Waltham, MA) with the excitation and emission wavelengths of 560 and 600 nm, respectively. To focus on the difference in the fluorescence of the prion-affected and control samples, the fluorescence index (FI) was calculated as follows: $FI = (Intensity_{prion} - Intensity_{control})/Intensity_{control} \times 100$ (%). The FI was indicated by an absolute value. Column statistical analyses were used to verify the results obtained. An FI at least 1.5 times higher than the 99% confidence interval value was considered significant. Fluorescence intensities of HPP18, HPP19, and HPP25 were minuscule, and therefore, the values were excluded from analyses.

2.4. Peptide pull-down assay

Biotinylated peptides were attached to streptavidin-coated magnetic beads (M-280; Veritas, Tokyo, Japan) for 2 h in TNB buffer (10 mM Tris, 150 mM NaCl, 0.1% BSA, pH 7.4). Beads were washed with TNB buffer, and then mixed with mouse, sheep, or cattle brain homogenate for 2 h at room temperature. Reactants bound to beads were magnetically pulled down. Beads were washed with TNB buffer containing 1% Triton X-100. They were then treated with or without 40 μg/ml of proteinase K (PK) at

Table 1
Fluorescent-peptide assay results.

Peptide ID	No. of residues[b]	Amino acid sequence[c,d]	Fluorescent-peptide assay[a]			
			Mouse	Sheep	Cattle	
			Scrapie		C-BSE	L-BSE
HPP01	1–12	MANLGSWMLVLFKKR	–	–	+	–
HPP02	11–25	LFVATWSDLGLSKKR	–	–	+	–
HPP03	21–35	LSKKRPKPGGWNTGG	–	–	–	+
HPP04	31–45	WNTGGSRYPGQGSPG	–	–	–	–
HPP05	41–55	QGSPGGNRYPPQGGG	–	–	–	–
HPP06	51–65	PQGGGGWGQPHGGGW	+	+	–	–
HPP07	61–75	HGGGWGQPHGGGWGQ	–	–	–	–
HPP08	71–85	GGWGQPHGGGWGQPH	–	–	–	–
HPP09	81–95	WGQPHGGGWGQGGGT	–	–	–	–
HPP10	91–105	QGGGTHSQWNKPSKP	–	–	–	–
HPP11	101–115	KPSKPKTNMKHMAGA	–	–	–	+
HPP12	111–125	HMAGAAAAGAVVGGL	–	–	–	–
HPP13	121–135	VVGGLGGYVLGSAMS	–	–	–	–
HPP14	131–145	GSAMSRPIIHFGSDY	–	–	–	–
HPP15	141–155	FGSDYEDRYYRENMH	–	–	–	–
HPP16	151–165	RENMHRYPNQVYYRP	–	–	–	–
HPP17	161–175	VYYRPMDEYSNQNNF	–	–	–	–
HPP18	171–185	NQNNFVHDSVNITIK	n.d	n.d	n.d	n.d
HPP19	181–195	NITIKQHTVTTTTKG	n.d	n.d	n.d	n.d
HPP20	191–205	TTTKGENFTETDVKM	–	–	–	–
HPP21	201–215	TDVKMMERVVEQMSI	–	–	–	–
HPP22	211–225	EQMSITQYERESQAY	–	–	–	–
HPP23	221–235	ESQAYYQRGSSMVLF	–	–	–	–
HPP24	231–242	SMVLFSSPPVILKKR	–	–	–	–
HPP25	242–253	LLISFLIFLIVGKKR	n.d	n.d	n.d	n.d
HPP06-s		QGGWPPGQGWGGHGG	–	n.d	n.d	n.d

[a] +, Significant; –, not significant; n.d, not determined.
[b] The number of residues is equivalent to human PrP sequence.
[c] Cysteine residue was substituted by serine residue for further application (double underlined).
[d] The basic amino acid residues (K and R) are underlined.