Proteolytic fragmentation of the murine prion protein: role of Tyr-128 and His-177

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Abstract The prion protein (PrP) has been proposed to display sequence and structural similarities to membrane-anchored signal peptidases [Glockshuber et al. (1998) FEBS Lett. 426, 291–296]. We have investigated the role of Tyr-128 and His-177 in the proteolytic fragmentation of murine PrP by mutating these residues to Phe and Leu, respectively, and expressing the resultant mutants in the human neuroblastoma SH-SY5Y. Both PrP-Y128F and PrP-H177L were expressed at the cell surface as glycosyl-phosphatidylinositol-anchored forms and were localised in detergent-insoluble membrane domains similar to wild type PrP. Following deglycosylation, the 19 kDa proteolytic fragment PrP-II was present in cells expressing either mutant, indicating that Tyr-128 and His-177 are not involved in the proteolytic fragmentation of PrP.

Key words: Transmissible spongiform encephalopathy; Proteolysis; Glycosyl-phosphatidylinositol anchor; Serine protease; Signal peptidase

1. Introduction

Prion diseases are a group of neurodegenerative diseases that include Creutzfeldt–Jakob disease, Gerstmann–Straussler–Scheinker syndrome, fatal familial insomnia and kuru in humans, scrapie in sheep and bovine spongiform encephalopathy in cattle. These diseases are caused by a conformational change in the normal cellular isoform of the prion protein (PrP^C) to the scrapie isoform PrP^{Sc} [1,2]. Human PrP^C is synthesised as a protein of 254 amino acids which undergoes a variety of post-translational modifications including glycosylation, glycosyl-phosphatidylinositol (GPI) anchor addition, and proteolysis [3]. The GPI anchor predisposes PrP^C to cluster in detergent-insoluble, cholesterol- and glycosphingolipid-rich membrane domains (DIGs) in the plasma membrane [4,5]. It is within DIGs that the fate of PrP^C is determined [6]. Either PrP^C undergoes a conformational change from a form with predominantly α-helical secondary structure to one with mainly β-sheet forming PrP^{Sc}, or it is proteolytically cleaved within the central hydrophobic, neurotoxic region (residues 106–126) generating an N-terminally truncated fragment termed PrP-II, which is then further metabolised [7–10]. Recombinant murine PrP (residues 23–231) expressed in *Escherichia coli* was also found to be sensitive to proteolytic degradation, with cleavage occurring after residues 116, 118 and 120 [11,12]. Prolonged incubation at acidic pH resulted in complete degradation of the N-terminal fragment (residues 23–120) while the C-terminal fragment was resistant to further metabolism.

Recently it was proposed that PrP^C has structural similarities to membrane-anchored signal peptidases, and that because of these similarities the proteolytic fragmentation of PrP^C may be due to autocatalysis [13]. Murine PrP (residues 121–217) is 22.7% identical and 40.9% similar to residues 49–137 of a catalytic subunit of rat signal peptidase which is a member of a larger clan (clan SF) of bacterial and eukaryote peptidases that have a Ser/Lys or Ser/His catalytic dyad [14,15]. Members of this clan include the *E. coli* umuD′ protein involved in SOS mutagenesis, the bacterial LexA protein involved in the control of DNA repair, as well as signal peptidases and mitochondrial/chloroplast leader peptidases which remove N-terminal peptides from secretory and organelle proteins, respectively. LexA and umuD′ both undergo autocatalysis; in the case of LexA this cleavage inactivates it, whereas umuD′ is activated [16]. Members of clan SF have a Ser residue which acts as the nucleophile, and either a Lys or His as the general base to increase the nucleophilicity of the active site Ser. In the *Pseudomonas* 7A glutaminase-asparaginase and *Thermoplasma acidophilum* 20S proteasome a Thr replaces the Ser [14]. Comparison of PrP with members of clan SF has shown that His-177 aligns with the conserved His/Lys, while in place of the Ser is another hydroxylated residue, Tyr-128 [13]. Both of these residues are completely conserved in PrP from all species.

We have investigated the hypothesis proposed by Glockshuber et al. [13] that Tyr-128 and His-177 are involved in the autocatalytic fragmentation of murine PrP by mutating these residues to Phe and Leu, respectively. The resulting mutant proteins have been expressed in the human neuroblastoma SH-SY5Y cell line, which lacks detectable levels of human PrP. The Y128F and H177L PrP mutants were both expressed at the cell-surface as glycosylated, GPI-anchored forms indistinguishable from wild type PrP and were localised in DIGs. In addition, both mutants underwent proteolytic fragmentation identical to that observed for the wild type protein, indicating that Tyr-128 and His-177 are not involved in this process.

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Abbreviations: DIG, detergent-insoluble, cholesterol- and glycosphingolipid-rich membrane domain; GPI, glycosyl-phosphatidylinositol; MBS, MES-buffered saline; PI-PLC, phosphatidylinositol-specific phospholipase C; PNGase F, peptide *N*-glycosidase F; PrP, prion protein; PrP^C, normal cellular form of PrP; PrP^{Sc}, scrapie isoform of PrP; PVDF, poly(vinylidene difluoride)

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2. Materials and methods

2.1. Generation of PrP mutants

Murine PrP cDNA with the 3F4 epitope tag (a gift from Dr D.A. Harris, Washington University, St. Louis, MO, USA) was subcloned using *Eco*RV into the mammalian expression vector pIRESneo (Clontech, California, USA). Directed mutagenesis was performed using the Stratagene Quick Change Mutagenesis Kit according to the manufacturer's instructions. The primers used to generate PrP-Y128F and PrP-H177L were 5′-GCT CCC CAG CAT CAA GCC AAG GCC-3′ and 5′-CAT ATT GAC GCA GTC GAG CAC GAA GTT GTT CTG G-3′, respectively. The mutants generated were confirmed by DNA sequencing.

2.2. Cell culture and transfection

SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum and 50 U/ml penicillin-streptomycin. Cells were maintained in 5% CO_2/95% O_2 at 37°C. Cells at mid-confluency were electroporated with either wild type DNA or one of the mutant sequences (30 μg cDNA in 0.8 ml) and then transferred to 80 cm^2 flasks. Stably expressing transfectants were selected 48 h post-transfection with 0.5 mg/ml Geneticin. Cell lysates were prepared from one 175 cm^2 flask of cells which was washed twice with phosphate-buffered saline (PBS) and then scraped into 25 ml PBS. Following centrifugation at $1000\times g$ for 3 min, the cell pellet was resuspended in 0.3 ml lysis buffer (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.5% sodium deoxycholate, 0.5% Triton X-100, 10 mM EDTA and protease inhibitor cocktail [Sigma, Poole, UK]) and incubated at room temperature for 30 min. The resulting lysates were clarified by centrifugation at $13\,000\times g$ for 5 min.

2.3. SDS-PAGE and Western blot analysis

Samples were mixed with an equal volume of reducing electrophoresis sample buffer (125 mM Tris–HCl, pH 6.8, 2% (w/v) SDS, 20% (v/v) glycerol, 100 mM dithiothreitol, bromophenol blue) and boiled for 3 min. Proteins were resolved by SDS polyacrylamide gel electrophoresis using a 15% acrylamide gel and transferred to Immobilon P poly(vinylidene difluoride) (PVDF) membranes [17]. The membranes were blocked by incubation in PBS containing 0.1% (v/v) Tween 20, 5% (w/v) dried milk powder, and 2% (w/v) bovine serum albumin overnight at 4°C. All primary and secondary antibody incubations were performed in the same buffer as that used for blocking. PrP was detected using antibody 3F4 which recognises residues 109–112 [18] or antibody 6H4 which recognises residues 144–152 [19]. Bound antibody was detected using peroxidase-conjugated secondary antibodies in conjunction with an enhanced chemiluminescence detection system (Amersham Life Sciences, Little Chalfont, Buckinghamshire, UK). Protein was quantified using bicinchoninic acid [20] in a microtitre plate assay with bovine serum albumin as standard.

2.4. Enzyme treatments

For release of PrP with *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C (PI-PLC) (a gift from Dr M.G. Low, Columbia University, New York, NY, USA), cells were rinsed three times with Opti-MEM and then incubated for 3 h at room temperature in Opti-MEM containing 0.2 U/ml PI-PLC. The medium was then harvested, concentrated by methanol precipitation and the resulting pellet resuspended in lysis buffer. For digestion of PrP with proteinase K, cell lysates were incubated for 15 min at 37°C with 3.3 μg/ml proteinase K. The reaction was terminated by adding an equal volume of boiling reducing sample buffer and boiling for a further 3 min. For deglycosylation of PrP with peptide *N*-glycosidase F (PNGase F), cell lysates were made 20 mM with respect to sodium phosphate, pH 7.6, 50 mM with respect to EDTA, 5% (w/v) with respect to SDS and 5% (v/v) with respect to 2-mercaptoethanol. Samples were boiled for 5 min, diluted 5-fold with 1.25% (v/v) Triton X-100 and incubated at 37°C for 16 h with 1 U PNGase F.

2.5. Isolation of DIGs

Cells were homogenised by 20 strokes in a Dounce homogeniser at 4°C in 0.5 ml MES-buffered saline (MBS), pH 6.5, containing 1% Triton X-100. Samples were mixed with an equal volume of 80% sucrose in MBS containing 1% Triton X-100 and a 1 ml aliquot injected under a 4 ml 5–30% sucrose gradient in MBS containing 0.5% Triton X-100. DIGs were isolated by centrifugation at $100\,000\times g$ for 18 h at 4°C [21]. After centrifugation the gradients were fractionated into 0.5 ml aliquots and the pellet resuspended in 0.5 ml MBS.

3. Results

Residues Tyr-128 and His-177 in murine PrP were mutated to Phe and Leu, respectively. Like the wild type PrP, the resulting mutants PrP-Y128F and PrP-H177L were epitopically tagged by replacement of Leu-108 and Val-111 with methionines which allows for their recognition by the species-specific antibody 3F4 [18]. Murine PrPs tagged with the 3F4 epitope have been used extensively in cell biological studies [22–24]. Each of the constructs was stably expressed in the human neuroblastoma SH-SY5Y [25], which lacks detectable levels of endogenous human PrP (Fig. 1). Cell lysate and medium samples from untransfected and transfected SH-SY5Y cells were then examined for the expression of PrP by Western blot analysis (Fig. 1). No endogenous human PrP was detected in untransfected SH-SY5Y cells. Wild type PrP and both of the mutants appeared as diffuse bands of 29–35 kDa, detectable only in the cell lysate and not in the medium.

To demonstrate that the wild type and mutant forms of PrP were GPI-anchored and present at the cell surface, intact cells were incubated with *B. thuringiensis* PI-PLC (Fig. 1). In the absence of PI-PLC, all of the PrP in the wild type and the two mutants was detected in the cell lysate, whereas in the presence of PI-PLC the majority of wild type PrP and virtually all of PrP-Y128F and PrP-H177L were detected in the medium, indicating that all three were present on the cell surface in a GPI-anchored form. PrP^C, like other GPI-anchored proteins, is enriched in DIGs [6]. The distribution of wild type PrP and the two mutants in DIGs within the SH-SY5Y cells was determined by buoyant sucrose density gradient centrifugation. Analysis of the resulting gradient fractions revealed that both PrP-Y128F and PrP-H177L, like wild type PrP, were present primarily in fractions 4–6 of the sucrose gradients (Fig. 2). The majority of flotillin, a well-characterised marker protein of DIGs from neuronal tissue [26,27], was also present in fractions 4–6, demonstrating that these fractions were en-

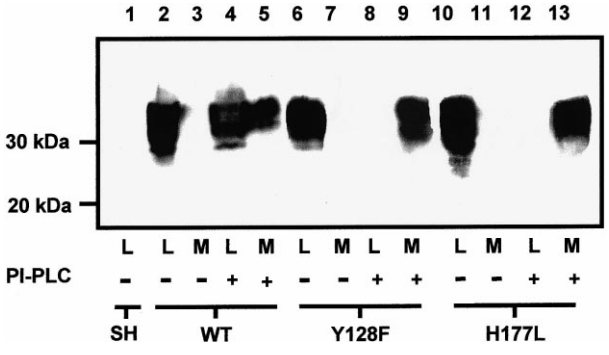

Fig. 1. Phospholipase C cleavage of wild type and mutant PrPs. Cell lysates (L, 10 μg of protein) and medium (M, 10 μg of protein) samples from either non-transfected SH-SY5Y cells (SH, lane 1) or cells expressing either wild type PrP (WT, lanes 2–5), PrP-Y128F (lanes 6–9) or PrP-H177L (lanes 10–13) were incubated as indicated in the absence or presence of *B. thuringiensis* PI-PLC before electrophoresis on a 15% acrylamide gel and then transfer to PVDF membrane. PrP was detected with antibody 3F4 as described in Section 2.

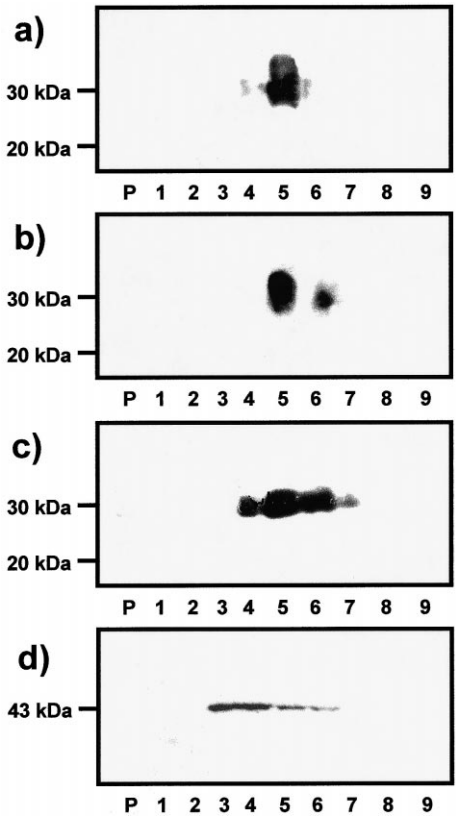

Fig. 2. Localisation of wild type and mutant PrPs in DIGs. SH-SY5Y cells expressing either (a and d) wild type PrP, (b) PrP-Y128F or (c) PrP-H177L were homogenised and the resulting homogenate injected under a 5–30% sucrose gradient. DIGs were separated from the solubilised protein by centrifugation as described in Section 2. The resulting sucrose gradients were fractionated from the top (fraction 9) to the bottom (fraction 1) and the pellet (P) was resuspended in MBS. Fractions were then electrophoresed on a 15% acrylamide gel, transferred to PVDF and either (a, b, c) PrP detected with antibody 3F4 or (d) flotillin detected with an anti-flotillin antibody (Transduction Laboratories, Lexington, KY, USA).

riched in DIGs. Consequently, neither mutation had affected the association of PrP with DIGs.

In order to assess whether the mutations had caused the acquisition of PrP^{Sc}-like properties, the proteinase K sensitivity of PrP-Y128F and PrP-H177L was assessed (Fig. 3). Following incubation with proteinase K, both wild type PrP and the two mutants were completely digested, with no evidence for a protease-resistant core indicative of PrP^{Sc}.

To characterise the proteolytic fragmentation of the wild type and mutant PrPs, cell lysates were digested with PNGase F in order to cleave the *N*-linked oligosaccharides from PrP. After deglycosylation, full-length PrP (27 kDa) and a 19 kDa proteolytic fragment, analogous to PrP-II observed in Syrian hamster brains [7], were detected in lysates from cells expressing wild type PrP using antibody 6H4 (Fig. 4). PrP-II is not recognised by antibody 3F4 and consequently represents a C-terminal fragment of PrP with its N-terminus lying in the region between the epitopes for the 3F4 and 6H4 antibodies [10]. PrP-II was also detected, to a similar extent as cells expressing wild type PrP, in cells expressing either PrP-Y128F or PrP-H177L, indicating that these mutations had no detectable effect on the proteolytic fragmentation of PrP when expressed in the SH-SY5Y cells.

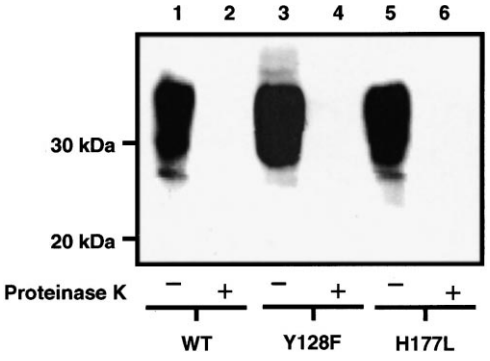

Fig. 3. Proteinase K digestion of wild type and mutant PrP. Cell lysates (10 μg of protein) from SH-SY5Y cells expressing either wild type PrP (WT, lanes 1 and 2), PrP-Y128F (lanes 3 and 4) or PrP-H177L (lanes 5 and 6) were incubated as indicated in the absence or presence of proteinase K before electrophoresis on a 15% acrylamide gel and then transfer to PVDF membrane. PrP was detected with antibody 3F4 as described in Section 2.

4. Discussion

Mutation of the active site Ser and Lys/His residues to Ala in members of clan SF inactivates these proteins [28–31], and in the case of Lex A and umuD′ prevents autocatalysis [16]. However, replacement of the active site Ser in *E. coli* LexA or *B. subtilis* signal peptidase with either Thr or Cys does not result in abolition of protease activity [29,30], indicating that other nucleophilic side chains can substitute for the hydroxyl group on Ser. Numerous proteases are known which have either a nucleophilic Ser or Cys involved in catalysis, while a limited number of examples use Thr [32]. To our knowledge no protease has to date been identified that has a catalytic Tyr, although the possibility of such enzymes existing cannot be ruled out, with PrP being the forerunner of a protease family with a novel catalytic mechanism.

In order to examine whether Tyr-128 and His-177 in PrP correspond to the active site Ser and Lys/His residues in members of clan SF and are involved in autocatalysis, thus testing the hypothesis of Glockshuber et al. [13], we mutated these

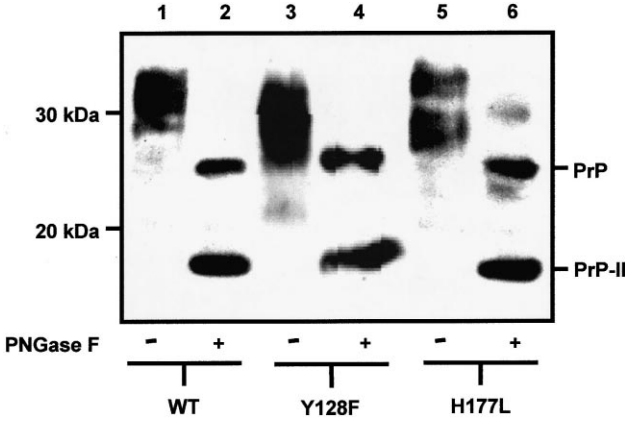

Fig. 4. Deglycosylation of wild type and mutant PrP. Cell lysates (10 μg of protein) from SH-SY5Y cells expressing either wild type PrP (WT, lanes 1 and 2), PrP-Y128F (lanes 3 and 4) or PrP-H177L (lanes 5 and 6) were incubated as indicated in the absence or presence of PNGase F before electrophoreses on a 15% acrylamide gel and then transfer to PVDF membrane. PrP was detected with antibody 6H4 as described in Section 2.

residues to Phe and Leu, respectively. The resulting mutants, when expressed in SH-SY5Y cells, were glycosylated, GPI-anchored and targeted to DIGs in the plasma membrane in an identical manner to wild type PrP. The mutant forms of PrP were still proteolytically processed to the 18 kDa fragment PrP-II, thus indicating that the observed proteolytic fragmentation is not an autocatalytic process involving Tyr-128 and His-177. Whether PrP has other, yet to be identified, proteolytic activity awaits to be determined, but the availability of the Tyr-128 and His-177 mutants should aid in this search. Alternatively, PrP may be catalytically inactive as a protease, with the three-dimensional structural similarities to the signal peptidases reflecting the fact that PrP binds to peptides or proteins without cleaving them. This would be analogous to the binding of peptides with C-terminal basic residues to the catalytically inert form of trypsin, anhydrotrypsin [33].

Given the current interest in PrP due to its role in transmissible spongiform encephalopathies such as new variant Creutzfeldt′Jakob disease in humans, and our limited understanding of the physiological roles of the protein, further investigation on the structural similarities between PrP and signal peptidases appear warranted.

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