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# Possible involvement of calpain-like activity in normal processing of cellular prion protein

Naomi Hachiya*, Yuko Komata, Sana Harguem, Kana Nishijima, Kiyotoshi Kaneko

*Department of Neurophysiology, Tokyo Medical University, 6-1-1 Shinjuku, Tokyo 160-8402, Japan*

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ABSTRACT

Time-lapse imaging analysis was previously used to show that spontaneous proteolysis of $PrP^C$, which is fluorescence-labeled at both $NH_2$- and COOH-termini, occurred in mouse neuroblastoma neuro2a (N2a) cells susceptible to $PrP^{Sc}$. We demonstrated that, unlike other protease inhibitors, a calpain inhibitor, calpastatin, drastically inhibited endoproteolysis of $PrP^C$, as observed with time-lapse imaging in living cells, suggesting calpain-like activity. Calpastatin also inhibited cleavage of endogenous $PrP^C$, and unprocessed molecules and the double-labeled $PrP^C$ accumulated around the perinuclear region. The molecular weight of $PrP^C$ fragments generated by spontaneous proteolysis was identical to those produced when $PrP^C$ synthesized *in vitro* was exposed to exogenous calpain. These results suggest that a calpain-like activity mediates normal processing of $PrP^C$ in N2a cells.

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Prion protein ($PrP^C$) is a secretory protein synthesized in the co-translational translocation system on the endoplasmic reticulum (ER) membrane, moving to the Golgi apparatus before becoming localized at the cell surface membrane, where they are bound by a glycosylphosphatidylinositol (GPI)-anchor [27,3]. $PrP^C$ can subsequently be converted into one of two mutually exclusive forms: it can be cleaved as a normal cellular prion protein, or it can remain uncleaved and be converted into as an abnormal protein, $PrP^{Sc}$, on the cell membrane. Numerous studies have shown that the exposure of the COOH-terminal fragment on the cell surface is a physiologically important issue [28,29,12]. Furthermore, the formation and infection of $PrP^{Sc}$ has been associated with a number of prion diseases, such as kuru, Creutzfeldt–Jakob disease (CJD), Gerstmann–Straussler–Scheinker syndrome (GSS), and fatal familial insomnia (FFI) in humans. Thus, molecular mechanisms leading to enhanced cleavage of $PrP^C$ are of potential interest and importance in terms of modulating prion protein-associated diseases [30]. Initial degradation of $PrP^C$ involves endoproteolytic cleavage of the $NH_2$-terminal fragment to produce a COOH-terminal polypeptide which is found in lipid rafts [28]. The cleavage site is mapped at the amino acid residues between epitopes of the 3F4 antibody (amino acids 108/111 in mouse [Mo] PrP) and the 13A5 antibody (amino acid residue 138 in Mo PrP) [28,24,23]. Other groups also reported that cleavage of $PrP^C$ occurred between amino acids 114/117 in chickens (ch) PrP [9], and 110/113 in humans [5].
The $NH_2$-terminal fragment of the PrP functions as a putative targeting element [25,26] and is essential for both translocation to the cell membrane and modulation of endocytosis [22]. GFP-tagged versions of $PrP^C$ were found to be properly anchored at the cell surface, and the distribution pattern was similar to that of endogenous $PrP^C$, with labeling observed at the cell membrane and in an intracellular perinuclear compartment [14,16,21,15,11]. Using fluorescent $PrP^C$ double-labeled at both $NH_2$- and COOH-termini, we previously demonstrated microtubule-associated intracellular localization of the fluorescent $NH_2$-terminal $PrP^C$ fragment in mouse neuroblastoma neuro2a (N2a) cells [7,8], known to be susceptible to $PrP^{Sc}$ infection. At steady state levels, we detected $NH_2$-terminal fluorescent $PrP^C$ fragments predominantly in the intracellular compartments, whereas COOH-terminal fluorescent $PrP^C$ fragments were found mainly at the cell surface; full-length $PrP^C$ with merged colors were detected around the perinuclear region. Time-lapse imaging analysis in living cells identified kinesin-driven anterograde and dynein-driven retrograde movements of the fluorescent $PrP^C$ fragments [8]. Thus, fluorescent imaging with double-labeled $PrP^C$ allowed us to observe the metabolic fate of $NH_2$- and COOH-terminal $PrP^C$ fragments simultaneously and in real time. During these investigations, we noticed that calpastatin, a calpain inhibitor, affected the abundance ratio of $NH_2$-terminal, COOH-terminal, and uncleaved $PrP^C$. Subsequent time-lapse imaging analysis and conventional biochemical analysis in combination with various protease inhibitors revealed the possible involvement of calpain-like activity in the normal processing of $PrP^C$. This is the first report of real-time imaging of the protein cleavage process of $PrP^C$ and the results

* Corresponding author. Tel.: +81 3 3351 6141x324; fax: +81 3 3351 6544.
*E-mail address:* naomi@tokyo-med.ac.jp (N. Hachiya).

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suggest that calpain-like activity mediates normal processing of $PrP^C$.

Anti-PrP antibodies K1 (PrP 26–40), K3 (PrP 76–90), K9 (PrP 196–210) and PrP(9–22) are rabbit polyclonal antibodies raised against synthetic peptides of PrP. The anti-COOH-terminal goat polyclonal antibody M20 was purchased from Santa Cruz Biotechnology, the anti-DsRed antibody was purchased from Clontech, and the anti-GFP antibody was purchased from Sigma–Aldrich. Anti-3F4 monoclonal PrP antibody was purchased from Signet Laboratories, Inc. Calpastatin was purchased from TaKaRa Corp. Anti-Golgi rabbit polyclonal antibody GM130 was purchased from Abcam Inc., polyclonal β-COP antibody was purchased from Thermo Fisher Scientific. Protease inhibitors chymostatin, leupeptin, and pepstatin were purchased from the Peptide Institute, Inc., while E-64 was purchased from Roche Applied Science. The inhibitors were used at the following concentrations: 0.7 μg/ml of pepstatin, 10 μg/ml of chymostatin, 10 μg/ml of leupeptin, 5 μg/ml of E64 and 50 μg/ml of calpastatin. MTT assay kit was purchased from Millipore (USA).

N2a cells were obtained from the American Tissue Culture Collection (ATCC). Cells were grown and maintained at 37 °C in MEM medium supplemented with 10% fetal bovine serum. N2a cells were transiently transfected with double-labeled constructs [7] using a DNA transfection kit (Lipofectamine 2000, Gibco BRL).

To observe living cells, cells were cultured on glass-bottomed dishes in culture medium without phenol red at 30 °C. Images of cells were collected with the Delta Vision Microscopy System (Applied Precision, Inc.) equipped with an Olympus IX70 through a cooled CCD camera. Fluorescent signals were visualized using a quad beamsplitter (Chroma), using excitation and emission filters of 525 nm and 550 nm, respectively.

Immunofluorescence microscopy observation was performed as previously described [6]. Briefly, cells were rinsed with PBS (+), fixed with 10% formalin in 70% PBS (+) for 30 min and incubated with 10% FBS containing PBS (−) for 30 min. After the addition of the first antibody (1:100 in PBS (−)) and incubation for 1 h, cells were washed with PBS (−) and incubated with the second antibodies, which was conjugated with Alexa488 or 594 for 2.5 h. The stained cells were washed four times with PBS (−) and mounted with Slow Fade Antifade Kit (Invitrogen). Samples were visualized with a Delta-Vision microscopy system (Applied Precision Inc). Out-of-focus light in the images was removed by interactive deconvolution.

Degradation experiments were carried out at 37 °C in the presence of 1 mM $CaCl_2$. Calpain I (Sigma) was added at a final concentration of 5 μg/ml to wheat germ-synthesized mouse full-length PrP (TNT T7 Quick Coupled Transcription/Translation System, Promega). Aliquots of the reaction mixture were sampled at various time points, and the reaction was stopped by the addition of electrophoresis sample buffer containing 1% sodium dodecyl sulfate (SDS). SDS-PAGE and immunoblot analysis using anti-K1 (PrP 26–40) antibody was carried out on all samples, and visualized by ECL Plus (GE Healthcare).

In control cells, we detected the DsRed-fused $NH_2$-terminal $PrP^C$ fragment (red) predominantly in intracellular compartments in a punctate distribution pattern, whereas GFP-fused COOH-terminal $PrP^C$ fragment (green) was mainly localized at the cell surface. Full length $PrP^C$ (yellow) was observed around the perinuclear region in real-time imaging identical to what was previously reported [7] (Fig. 1, Control). Using this double-labeled $PrP^C$ expression and real-time imaging analysis system, we examined the effect of various protease inhibitors on the cleavage of $PrP^C$. Among the protease inhibitors tested, a calpain inhibitor, calpastatin, strongly reduced proteolysis of fluorescent-labeled $PrP^C$. Full-length PrP gradually and drastically accumulated in a time-dependent manner, suggesting the specific involvement of calpain-like activity in the processing of $PrP^C$ (Fig. 1).

We also examined localization of endogenous $PrP^C$ with immunofluorescent microscopy using the anti-PrP polyclonal antibodies K3 and M20, both with calpastatin treatment. In agreement with the results of the time-lapse experiment of double-labeled PrP, calpastatin-treated cells exhibited distinct distribution profiles for the processed fragments and full-length $PrP^C$ (Fig. 2A). To identify the perinuclear region in which calpastatin-treated endogenous or the double labeled $PrP^C$ accumulated, we examined immunofluorescent microscopy analysis using anti-Golgi antibodies, GM130 or β-COP. As is clear in Fig. 2B, accumulated both of $PrP^C$ were merged with the Golgi markers, suggesting that $PrP^C$ was at least partly stacked in the Golgi membrane. Furthermore, *in vitro* (wheat germ) synthesized DsRed-PrP-GFP (WG/DsRed-PrP-GFP) was detected by anti-PrP(9–22) antibody which raised against the ER-targeting signal region, while the cell lysate from the DsRed-PrP-GFP expressed N2a was not (N2a/DsRed-PrP-GFP). Hence the artificial substrate (the DsRed-PrP-GFP) processed the ER-targeting signal and behaved like endogenous form of $PrP^C$ (Fig. 2C). Cell viability of the calpastatin-treated N2a was same as the control indicating that calpastatin treatment did not affect the cell death (Fig. 2D). All together, these data exclude the possibility of an artificial distribution of fluorescent proteins, confirmed by the results in Fig. 1.

Western blot analysis was also performed to examine the biochemical profiles of calpain-processed $NH_2$-terminals, COOH-terminals, and full-length $PrP^C$. In the absence of calpastatin, $NH_2$-terminal fragments from DsRed-tagged PrP (∼36 kDa, Lane 1, arrowhead in Fig. 3A) and endogenous PrP $NH_2$-terminal fragments (∼10 kDa, Lane 1, arrowhead in Fig. 3B) were detected with anti-DsRed and anti-K1 antibodies, respectively. Similarly, the GFP-tagged COOH-terminal fragments of PrP (∼46 kDa, Lane 3, arrowhead in Fig. 3A) and endogenous COOH-terminal fragments of PrP (∼20 kDa, Lane 3, arrowhead in Fig. 3B) were detected with anti-DsRed and anti-K9 antibodies, respectively. In contrast, in the presence of calpastatin, the cleavage of PrP was inhibited and full-length double-labeled and endogenous PrPs were detected as molecules of molecular weight of ∼76 kDa and ∼30 kDa, respectively (Lanes 2 and 4, arrows in Fig. 3A and B). These results verify the real-time imaging analysis of PrP processing described above.

To further evaluate calpain-like activity for the processing of $PrP^C$, we used an *in vitro* assay to test if PrP was a proteolytic substrate for calpain-dependent degradation. Wheat germ-synthesized full-length PrP was exposed to calpain, which resulted in degradation. In the absence of calpain, no PrP degradation was observed (Fig. 4, Lane 1). Calpastatin and the calcium chelator, EGTA, prevented calpain activation, thus preventing PrP cleavage *in vitro* (Fig. 4, Lanes 2 and 3). The degradation was limited; a number of proteolytic products accumulated in the reaction mixture, with no further degradation seen after 180 min (Fig. 4, Lanes 4–7). This finding is in accordance with the fact that calpain usually degrades substrates to a limited extent [6]. Thus, the degradation of *in vitro*-synthesized PrP provides conclusive evidence that PrP is a substrate for calpain and is compatible with previous reports [28,24,23].

Time-lapse imaging analysis detected the aberrant proteolytic cleavage of double-labeled fluorescent $PrP^C$ in a calpain-like activity-dependent manner (Fig. 1), which disrupts the neurotoxic and amyloidogenic region of $PrP^C$ comprising residues 106–126 [28,24,23]. Although some $PrP^C$ was normally cleaved and the COOH-terminal fragment was located at the cell membrane even in the presence of calpastatin (Fig. 1, calpastatin), this may have occurred for two reasons: (1) calpastatin did not completely block calpain-like activity in the cell; or (2) a calpastatin-insensitive protease rescued the processing and translocation of the COOH-

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