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# Proteolytic processing of the ovine prion protein in cell cultures

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**Abstract**

The cellular compartment and purpose of the proteolytic processing of the prion protein (PrP) are still under debate. We have studied ovine PrP constructs expressed in four cell lines; murine neuroblastoma cells (N2a), human neuroblastoma cells (SH-SY5Y), dog kidney epithelial cells (MDCK), and human furin-deficient colon cancer cells (LoVo). Cleavage of PrP in LoVo cells indicates that the processing is furin independent. Neither is it reduced by some inhibitors of lysosomal proteinases, proteasomes or zinc-metalloproteinases, but incubation with bafilomycin $A_1$, an inhibitor of vacuolar $H^+$/ATPases, increases the amount of uncleaved PrP in the apical medium of MDCK cells. Mutations affecting the putative cleavage site near amino acid 113 reveal that the cleavage is independent of primary structure at this site. Absence of glycosylphosphatidylinositol anchor and glycan modifications does not influence the proteolytic processing of PrP. Our data indicate that PrP is cleaved during transit to the cell membrane.


*Keywords:* Prion protein; Proteolytic processing; Cell culture; Ovine

A key molecular event in prion diseases, such as Creutzfeldt–Jakob disease in human and scrapie in sheep, is the poorly understood conversion of normal cellular glycosylphosphatidylinositol (GPI)-anchored prion protein ($PrP^C$, herein referred to as PrP) into a malformed and dysfunctional conformer ($PrP^{Sc}$) that is partly proteinase resistant, reviewed in [1]. In normal brain and several other tissues, as well as in cultured cells, PrP is to varying degrees proteolytically processed near amino-acid 113 (numbering according to ovine PrP), N-terminal to a hydrophobic region of the protein [2–5]. This highly consistent proteolytic processing of PrP separates the structurally disordered $Cu^{2+}$-binding domain of the protein from the globular C-terminal two-thirds. The precise cellular site of this cleavage and its possible role for the cellular function of PrP are still a matter of controversy. Concerning the cellular compartment for the cleavage, however, the consensus appears to be that PrP is processed during cycling between the plasma membrane and endosomes, and that there are at least two possible cleavage sites within the N-terminal portion of PrP [3–6]. For a detailed analysis of the heterogeneity of brain PrP, see [7]. The major cleavage, which is the focus of this work, has been shown to destroy the epitope for the monoclonal antibody 3F4 [5]. This epitope is naturally present in human and hamster PrP, and has an absolute requirement for the sequence MKHM (109–112) immediately N-terminal to the hydrophobic stretch of PrP [8]. Sequence analysis of fragmented PrP derived from human neuroblastoma cells has indicated that the major cleavage site resides in the C-terminal portion of the 3F4 epitope [5], which corresponds to amino-acids 112–115 in the ovine PrP. Cleavage of hamster PrP expressed in N2a cells was shown to be insensitive to neutralisation of acidic compartments by $NH_4Cl$ treatment, suggesting that this is an event distinct from the general breakdown of PrP in lysosomes [9]. However, studies of chicken PrP expressed in N2a cells indicated that inhibition of lysosomal

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proteases by various agents, including $NH_4Cl$, reduced the liberation of N-terminal cleavage products into the culture medium [4]. Since GPI-anchored proteins appear to cluster into dynamic microdomains, rafts, of the lipid bi-layer, it has been suggested that the cleavage of PrP could be performed by proteinases specifically localised to such membrane domains [9]. More recently, it has been suggested that the fibrinolytic serine proteinase plasmin can be involved in the cleavage of PrP [10,11].

The general breakdown of PrP in crude human brain homogenate was strongly inhibited by metal chelators such as EDTA and EGTA, an effect that could be directly reversed by addition of $Fe^{2+}$ and $Cu^{2+}$ ions. Other classes of proteinase inhibitors had minor effects on the breakdown of PrP [12]. Interestingly, inhibition of the disintegrins ADAM10 (a disintegrin and metalloproteinase) and TACE (tumor necrosis factor α converting enzyme, ADAM17) reduced the liberation of the N-terminal portion of PrP to the cell culture medium of human embryonic kidney cells [13], pointing to a possible role for these zinc metalloproteinases in the proteolytic processing of PrP. Another study found that an α-secretase like activity, possibly raft-associated, can be involved in phorbol ester-stimulated shedding of full-length PrP by cleaving the extreme C-terminus of the PrP [14].

Most studies of the proteolytic processing of PrP in cell culture have focused on C-terminal PrP fragments in cell lysates and N-terminal fragments in conditioned media. The fate of the N-terminal tail has proven difficult to study, not least due to its size, which is somewhere between 7 and 9 kDa. To circumvent this problem we have generated several PrP constructs carrying green fluorescent protein (GFP) within the N-terminal portion of the protein. Similar PrP::GFP chimaeras have been used by a number of other groups [15–18]. In general, the cellular processing and behaviour of PrP when fused with GFP in this way appear normal. Herein, we compare the proteolytic processing of PrP::GFP fusion proteins in different cell lines and in the presence of different proteinase inhibitors. Our data indicate that the processing of PrP::GFP fusion proteins occurs according to the same pattern as for un-tagged PrP. Furthermore, this processing appears insensitive to mutations affecting the proposed major cleavage site and is independent of GPI anchoring and glycan modifications of the protein. However, treatment with bafilomycin $A_1$ (Baf $A_1$), an inhibitor of vacuolar $H^+$/ATPases, allowed more uncleaved PrP to be detected, particularly in the apical medium of MDCK cells. Interestingly, the processing of amyloid precursor protein (APP) by β-secretase (BACE 1), which is of major importance in Alzheimer's disease, is also reduced by Baf $A_1$ [19]. Our data suggest that PrP is constitutively processed, possibly in a slightly acidic compartment during transit through the secretory pathway, en route to the plasma membrane.

## Materials and methods

### *Sheep brain tissue*

Crude preparations of PrP-rich microsomal and synaptosomal membranes from sheep brain were produced as described [20]. Briefly, immediately after euthanasia with pentobarbital and exsanguination through the carotid arteries, 40 g of brain tissue was homogenised in 200 ml ice-cold buffer containing 0.32 M sucrose, 0.5 mM KCl, 1 mM $Mg_2Cl$, 1 mM $NaHCO_3$, 1 mM EDTA supplemented with proteinase inhibitor tablets (Complete, Roche), and 1 mM dithiothreitol (DTE). The homogenate was centrifuged at 3000*g* for 10 min at 4 °C. The pellets were rehomogenised in 170 ml of the same buffer and centrifuged at 3000*g* for 10 min at 4 °C. Supernatants were centrifuged through a 0.85 M sucrose cushion at 100,000*g* for 1 h at 4 °C. The pellets were resuspended in lysis buffer (LB) containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.5% (v/v) Triton X-100, 0.5% sodium deoxycholate, and 1 mM EDTA, supplemented with Complete proteinase inhibitor tablets (Roche).

### *DNA constructs*

The primers used in cloning of the PrP constructs are shown in Table 1.

*PrP-pGEM T-Easy*. The ovine prion protein was initially cloned into the pGEM-T Easy vector (Promega) using the Expand Long Template PCR System (Roche), primer 1 and 2, and genomic DNA as template.

*PrP-pcDNA3*. The coding region of PrP was amplified from PrP-pGEM T-easy using primer 3 and 4 containing an *Eco*RI and a *Xho*I restriction site, respectively, and cloned into pGEM-T Easy. PrP was cut out with the restriction enzymes *Eco*RI and *Xho*I, and ligated into the corresponding restriction sites of the expression vector pcDNA3 (Invitrogen).

*PrP::GFP-pcDNA3*. A GFP-labelled PrP was constructed by ligating GFP into the *Xma*I site of PrP-pGEM-T Easy. GFP was amplified from pEGFP-C1 (Clontech) using primer 5 and 6 containing *Xma*I sites, and cloned into pGEM-T Easy. After *Xma*I digestion of the plasmid, GFP was ligated into *Xma*I digested PrP-pGEM-T Easy. The restriction enzymes *Eco*RI and *Xho*I were used to transfer the PrP-GFP from pGEM T-Easy to pcDNA3.

*PrP::GFPΔGPI-pcDNA3*. A secretory PrP::GFP was constructed by introducing a stop codon at the GPI-anchoring site (Ser234Stop) using PrP::GFP-pcDNA3 as template, and primer 7 and 8 containing an *Eco*RI and a *Xho*I restriction site, respectively. The amplification product was cloned into pGEM-T Easy. PrP::GFPΔGPI was cut out with *Eco*RI and *Xho*I, and ligated into pcDNA3.

All DNA constructs were sequenced to confirm correct amplification and cloning.

### *Site-directed mutagenesis*

Mutations were generated by PCR-mediated mutagenesis in PrP::GFP-pcDNA3 using QuickChange II Site-directed Mutagenesis Kit (Stratagene). Mutation V115M introduced the human and hamster epitope 3F4 into the ovine PrP::GFP. The forward (F) and reverse (R) primers used in the site-directed mutagenesis are shown in Table 1. All mutations were verified by sequencing.

### *Cell cultures*

Madin–Darby canine kidney (MDCK II) cells were grown in DMEM (Dulbecco's modified Eagle's medium, Cambrex) with addition of 1% glutamine (Cambrex) and 5% foetal bovine serum (FBS, PAA, Australia). The neuroblastoma cell lines N2a (murine) and SH-SY5Y (human) were kindly provided by Dr. Jörg Tatzelt (Max Planck Institute of München, Germany). N2a cells were grown in minimum essential medium (MEM, Sigma) added 10% FBS (Euroclone), non-essential amino-acids, and pyruvate (Cambrex). SH-SY5Y were grown in DMEM with 10% FBS (Euroclone). Human furin-deficient colon cancer cells (LoVo) transfected