Scrapie-like prion protein is translocated to the nuclei of infected cells independently of proteasome inhibition and interacts with chromatin

Alain Mangé*, Carole Crozet*, Sylvain Lehmann and Florence Béranger‡

Institut de Génétique Humaine, UPR CNRS1142, 141 Rue de la Cardonille 34396 Montpellier CEDEX 5, France

*These authors contributed equally to this work
‡Author for correspondence (e-mail: florence.beranger@igh.cnrs.fr)

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Summary

Prion diseases are fatal transmissible neurodegenerative disorders characterized by the accumulation of an abnormally folded isoform of the cellular prion protein (PrP<sup>C</sup>) denoted PrP<sup>Sc</sup>. Recently, wild-type and pathogenic PrP mutants have been shown to be degraded by the endoplasmic reticulum-associated degradation proteasome pathway after translocation into the cytosol. We show here that a protease resistant form of PrP accumulated in the nuclei of prion-infected cells independently of proteasome activity, and that this nuclear translocation required an intact microtubule network. Moreover, our results show for the first time that nuclear PrP interacts with chromatin in vivo, which may have physiopathological consequences in prion diseases.

Key words: Prion, Nucleus, Microtubules

Introduction

Prion diseases are fatal, neurodegenerative disorders in humans and animals, which include Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep, and bovine spongiform encephalopathy (BSE) in cattle. These disorders are characterized by the generation of an abnormally folded isoform of the cellular prion protein (PrP<sup>C</sup>), denoted PrP<sup>Sc</sup>, which represents the major component of infectious prion particles (Prusiner et al., 1998). PrP<sup>C</sup>, a neuronal membrane glycoprotein whose function has not been fully characterized, is rich in α-helical regions. During the development of the disease, this protein can be converted into PrP<sup>Sc</sup>, a protease-resistant isoform rich in β-sheet content that accumulates in the brain of infected organisms (Cohen et al., 1994). It has been postulated that PrP<sup>Sc</sup> may physically interact with PrP<sup>C</sup>, acting as a template for the formation of new PrP<sup>Sc</sup> molecules.

Wild-type PrP<sup>C</sup> molecules can be subject to the endoplasmic reticulum-associated degradation (ERAD)-proteasome pathway (Ma and Lindquist, 2001; Yedidia et al., 2001), in the course of which they are mislocated in the cytosol. Accumulation of cytosolic PrP molecules is highly toxic and induces neuronal cell death (Ma et al., 2002). In prion-infected cells, PrP is subject to retrograde transport from the plasma membrane towards the ER, and this compartment may play a significant role in PrP<sup>Sc</sup> conversion (Beranger et al., 2002). Presence of PrP<sup>Sc</sup> in the ER may lead to an accumulation of misfolded PrP molecules which could be subsequently mislocated in the cytoplasm. Interestingly, PrP contains amino-proximal nuclear localization signals (NLS) (Basler et al., 1986; Gu et al., 2003) and has been detected previously in the nuclei of prion-infected cells by immunofluorescence studies (Pfeifer et al., 1993). Some pathogenic mutant prion proteins have been also detected in the nucleus following proteasome inhibition (Lorenz et al., 2002; Zanusso et al., 1999). Therefore, it is possible that cytosolic PrP accumulates in the nucleus when the proteasome function is altered, and the presence of nuclear PrP has deleterious effects and important pathological consequences.

To test whether the nuclear localization of PrP is due to an alteration of proteasome activity, we studied the effect of proteasome inhibitors on nuclear accumulation of PrP in non-injected or prion-infected N2a cells. Several neuronal cell lines persistently infected with mouse-adapted scrapie have been developed for investigation of the biochemical properties of PrP<sup>Sc</sup> (Butler et al., 1988; Race et al., 1987). In this study we used a mouse neuroblastoma cell line (N2a) infected by the 22L prion strain, whose generation has been described (Nishida et al., 2000) (see Materials and Methods). In this system, we detected PrP molecules in the nuclei of prion-infected cells only, independently of proteasome activity. This nuclear PrP has the biochemical properties of PrP<sup>Sc</sup> and we observed that its transport to the nucleus required an intact microtubule network. Furthermore, we showed that the nuclear PrP was associated with chromatin in vivo.

Materials and Methods

Cells, buffers and reagents

N2a and ScN2a cells used in this paper correspond, respectively, to N2a#58 and N2a#58-22L cells described previously (Nishida et al., 2000). These cells have been obtained by infecting PrP-expressing N2a cells with the 22L mouse adapted scrapie strain, and produce PrP<sup>Sc</sup> molecules constitutively. The infected status of the cells was regularly assessed by proteinase K digestion analysis to confirm the presence of PrP<sup>Sc</sup>. Cell culture conditions and monoclonal antibodies used to improve PrP detection were described previously (Beranger et al., 2002). Other antibodies were from Santa Cruz Biotechnology (USA). All other reagents were from Sigma (France).
Immunofluorescence microscopy

ScN2a cells were fixed in 4% paraformaldehyde in PBS for 30 minutes, washed three times with PBS and permeabilised in 0.5% Triton X-100-PBS for 5 minutes. Permeabilised cells were treated for 5 minutes in 3M guanidine thiocyanate and further incubated overnight at 4°C with primary antibodies diluted in 5% milk in PBS. After three washes in PBS, cells were incubated with Rhodamine conjugated anti-mouse antibodies (dilution 1/300) for 1 hour at 37°C. Nuclei were stained with Hoechst 33286. Cells were washed and mounted in FluorSave reagent (Calbiochem). Images were collected and processed using a Leica microscope. For confocal analysis, images were obtained using a Leica laser-scanning microscope.

Preparation of nuclei

Pure nuclei from N2a or ScN2a cells were prepared with Pure Prep Nuclei Isolation Kit (Sigma, France) according to the manufacturer’s instructions. For immunofluorescence studies, a small volume of the final suspension of nuclei was placed onto polylysine-coated microscope slides and allowed to adhere for 2 hours at 4°C. The slides were then washed in PBS before being subjected to the immunofluorescence microscopy procedure described above.

For the deglycosylation experiment, nuclei lysates were treated with N-glycosidase F (0.01 units/ml) for 16 hours at 37°C before analysis.

Analysis of the nuclear association of the prion protein

For DNase, NaCl and EDTA treatments, pure nuclei from ScN2a cells were lysed for 10 minutes on ice in 200 μl of modified CSK buffer (10 mM Pipes pH 6.8, 100 mM NaCl, 300 mM sucrose, 1 mM MgCl2, 1 mM EGTA, 1 mM DTT, 1 mM PMSF and protease inhibitors) containing 0.1% Triton X-100 (0.1% Tx-CSK) (Blencowe et al., 1994). After low speed centrifugation, the nuclei were resuspended in 200 μl of 0.1% Tx-CSK. Aliquots were incubated at the indicated temperature in the presence of DNase, various concentration of NaCl or EDTA. For DNase digestion, the samples were supplemented with 1 mM MgCl2. After incubation, the pellet fraction and the soluble supernatant were separated by low speed centrifugation.

Results and discussion

Impairment of proteasome function induced accumulation of detergent-insoluble and protease-resistant PrP molecules in the cytoplasm (Ma and Lindquist, 2001; Yedidia et al., 2001). According to the authors, these PrP species correspond to proteins that failed to be degraded by the ERAD pathway. However, a recent study demonstrated that proteasome inhibitors increased the transcription level of mRNAs encoding PrP in transfected cells (Drisaldi et al., 2003). Under these conditions, a signal-peptide-bearing form of PrP accumulated on the cytoplasmic face of the ER membrane. In any case, treatment of cells with proteasome inhibitors induces the accumulation of cytosolic PrP species. Since mutant PrP have been described to translocate in the nucleus after proteasome inhibition (Lorenz et al., 2002; Zanusso et al., 1999), we wanted to determine whether in the same conditions cytosolic PrP molecules could also be transported to the nucleus. As already described (Ma and Lindquist, 2001; Yedidia et al., 2001), we observed that an overnight ALLN (N-acetyl-leucinal-leucinal-norleucinal) treatment of non-infected N2a cells induced the accumulation of PrP species into the cytoplasm of the cells (Fig. 1A). Similar results were obtained in the presence of lactacystin, another proteasome inhibitor in the presence of lactacystin, another proteasome inhibitor.
Scrapie-like prion protein in the nucleus

from the endoplasmic reticulum was verified by western blot analysis using anti-calnexin antibodies (Fig. 1D).

These results demonstrated clearly that the cytosolic protease-resistant PrP species induced by ALLN treatment in non-infected or prion-infected N2a cells and described in previous studies (Beranger et al., 2002; Cohen and Taraboulos, 2003; Ma and Lindquist, 2001; Yedidia et al., 2001) was not transported to the nucleus. However, PrPSc molecules accumulated in the nucleus of prion-infected cells, even if the proteasome function was not impaired. Deglycosylation using N-glycosidase digestion of nuclei extracts showed a shift in the PrPSc bands demonstrating that nuclear PrPSc was fully glycosylated (Fig. 1E), and thereby confirming that it is different from the unglycosylated cytoplasmic species. This glycosylated PrPSc could originate from retrograde transport within the ER as we have previously demonstrated (Beranger et al., 2002), or from a direct transport from the plasma membrane to the nucleus as described for some viral proteins (Harel and Forbes, 2001).

In order to confirm the presence of PrPSc in the nuclei of prion-infected cells, whole cells or isolated nuclei were analyzed by indirect immunofluorescence. The immunofluorescence detection of PrPSc requires denaturation of PrPSc molecules to enhance the binding of PrP antibodies (Taraboulos et al., 1990). Indeed, after optimal fixation and permeabilisation using paraformaldehyde and 0.1 Triton X-100, a 3M guanidine thiocyanate treatment was performed to expose epitopes that were previously hidden on PrPSc. Immunofluorescence microscopy studies were carried out using an antibody that is specific for the C-terminal extremity of PrPSc. In non-infected cells, no immunoreactive material could be detected (Fig. 2Aa). In prion-infected cells, most PrPSc was found to be intracellular and mainly localized to perinuclear structures reminiscent of the Golgi apparatus (Fig. 2Ab). However, detailed inspection of the cells revealed the presence of PrPSc nuclear localization in approximately 10% of the prion-infected cells (Fig. 2Ab, white arrows, and panels c-d). In those cells, 80-100% of the PrPSc is located in the nucleus. To demonstrate that the fluorescent signal really originated from the nuclear interior rather than from sources external to the nuclear envelope, staining was analysed by confocal laser-scanning microscopy. A confocal optical section along the same z-axis of prion-infected cells is shown in Fig. 2B and confirms the presence of PrPSc in the nucleus of some cells (white arrow). It is interesting to note that, in each case, the PrPSc signal seems to be concentrated in areas where the Hoechst label is less intense, which suggests that the protein is excluded from the heterochromatin zones and accumulates in euchromatin areas, a highly transcribed region of the nucleus. Indirect immunofluorescence experiments were also performed on isolated nuclei (Fig. 3Ba-c), and again PrPSc could be detected within the nucleus.

Accumulation of the cytoplasmic protease-resistant PrP molecules in the cytosol is not by itself sufficient to target the protein into the nucleus. Because the N-terminal nuclear localisation signal sequence of PrP is not efficient in localising the protein to the nuclear compartment (Jaegly et al., 1998), we considered that nuclear transport of PrPSc molecules in infected cells may involve a complex mechanism requiring a specific conformation of the protein or its association with accessory molecules. Since nucleocytoplasmic trafficking can involve cytoskeleton structures (Aplin et al., 2001), we determined whether PrP accumulation in the nucleus required either an intact actin cytoskeleton or a functional microtubule network. ScN2a cells were treated for 6 hours either with the actin depolymerizing agent cytochalasin D or the microtubule-disrupting drug colchicine. Colchicine treatment inhibited...
accumulation of PrP in the nucleus, whereas cytochalasin D treatment had no effect (data not shown). An overnight treatment of ScN2a cells with various concentrations of colchicine did not modify the amount of PrP in total cell lysates nor the proportion of PrPSc in PK treated cell extracts (Fig. 3A) while accumulation of PrP in the nucleus was strongly reduced in the presence of the highest concentrations of the drug. Immunofluorescence microscopy analysis showed that colchicine treatment of the cells decreased PrPSc labelling in the nuclei to background level (Fig. 3Bd-f). At the whole cell level, colchicine treatment induced the accumulation of the protein in large cytoplasmic inclusions (Fig. 3B, panels j-l).

These observations indicate that, as described for other proteins (Campbell and Hope, 2003; Giannakakou et al., 2000; Hanz et al., 2003; Lam et al., 2002), nuclear translocation of PrP requires a functional microtubule network, disruption of which results in reduced nuclear accumulation. In agreement with our results, it has been found that α and β tubulins bind the peptide PrP106-126 (Brown et al., 1998). Whether some PrP molecules are associated with cellular microtubules in infected cells and are transported to the nucleus by association with the microtubule-based motor proteins is currently under investigation.

Given the in vitro DNA-binding properties of recombinant PrP (Gabus et al., 2001a; Gabus et al., 2001b; Sklaviadis et al., 1993), we were interested to know how nuclear PrP would fractionate with respect to various chromatin extraction procedures. DNA-bound proteins can be released from chromatin by different treatments such as DNase and NaCl (Lichota and Grasser, 2001; Tatsumi et al., 2000). ScN2a cell nuclei were incubated with DNase I or increasing concentrations of NaCl (Fig. 4A,B). DNase treatment increased the proportion of nuclear PrP in the insoluble pellet fractions, suggesting that upon DNA digestion, PrP was released and aggregated. These results could be explained by a recent study showing that recombinant murine prion protein and prion peptides bound with high affinity to DNA sequences, and were maintained in a soluble form (Cordeiro et al., 2001). In this paper, the authors demonstrated that double-stranded DNA sequences completely inhibited aggregation of prion peptides, whereas DNA binding prevention induced their aggregation. These data are in agreement with our experiments in ScN2a cells nuclei: they suggest that nuclear PrP binds to DNA in vivo and that liberation of nuclear PrP from DNA induces aggregation of the protein into an insoluble form.

The extractability of nuclear PrP from chromatin in Triton X-100-treated nuclei was determined by incubation with increasing concentrations of NaCl (Fig. 4B). Most of nuclear PrP was still insoluble with 200 mM NaCl, whereas more than 90% of PrP became soluble with 400 mM NaCl. These results suggest that nuclear PrP is most likely associated with chromatin. DNA-protein interactions frequently require divalent cations such as Mg2+. To get further insight into the mechanism of DNA-nuclear PrP interaction, Triton X-100-extracted nuclei were supplemented with an excess of EDTA. In the presence of EDTA, PrP molecules were liberated from the nuclei (Fig. 4C). These findings suggest that divalent cations may play a role in the chromatin/DNA association of nuclear PrP. Whether Cu2+, Zn2+ or Mn2+, all of which have been shown to bind PrPSc, are required for chromatin association of PrPSc remains to be determined.

Whether nuclear PrP binds to specific nuclear proteins or DNA sequences in the nuclei of prion-infected cells is currently
under investigation. A search for candidate proteins binding to prion protein led, among other proteins, to the identification of Nrf2 (Yehiely et al., 1997), a key transcription factor that mediates Cu/Zn super-oxide dismutase (SOD1) induction in response to environmental stress (Park and Rho, 2002). A nuclear interaction between PrP and Nrf2 could have implications in the role played by oxidative stress in the neurodegeneration observed in TSEs, the presence of PrPSc in the nuclei of prion-infected cells could have potential pathological consequences. Nuclear PrPSc concentrates in euchromatin areas that correspond to a highly transcribed part of the nucleus. Several studies have shown that the in vitro interaction of PrP with nucleic acids results in the formation of nucleoprotein complexes (Gabus et al., 2001a; Murdoch et al., 1990). Such complexes could in vivo impede the function of transcription factors and alter gene expression, resulting in a cascade of biological processes that could result in prion pathogenesis.

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