Characterization of intracellular localization of PrP<sub>Sc</sub> in prion-infected cells using a mAb that recognizes the region consisting of aa 119–127 of mouse PrP

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Generation of an abnormal isoform of the prion protein (PrP<sub>Sc</sub>) is a key aspect of the propagation of prions. Elucidation of the intracellular localization of PrP<sub>Sc</sub> in prion-infected cells facilitates the understanding of the cellular mechanism of prion propagation. However, technical improvement in PrP<sub>Sc</sub>-specific detection is required for precise analysis. Here, we show that the mAb 132, which recognizes the region adjacent to the most amyloidogenic region of PrP, is useful for PrP<sub>Sc</sub>-specific detection by immunofluorescence assay in cells pre-treated with guanidine thiocyanate. Extensive analysis of the intracellular localization of PrP<sub>Sc</sub> in prion-infected cells using mAb 132 revealed the presence of PrP<sub>Sc</sub> throughout endocytic compartments. In particular, some of the granular PrP<sub>Sc</sub> signals that were clustered at peri-nuclear regions appeared to be localized in an endocytic recycling compartment through which exogenously loaded transferrin, shiga and cholera toxin B subunits were transported. The granular PrP<sub>Sc</sub> signals at peri-nuclear regions were dispersed to the peripheral regions including the plasma membrane during incubation at 20 °C, at which temperature transport from the plasma membrane to peri-nuclear regions was impaired. Conversely, dispersed PrP<sub>Sc</sub> signals appeared to return to peri-nuclear regions within 30 min during subsequent incubation at 37 °C, following which PrP<sub>Sc</sub> at peri-nuclear regions appeared to redisperse again to peripheral regions over the next 30 min incubation. These results suggest that PrP<sub>Sc</sub> is dynamically transported along with the membrane trafficking machinery of cells and that at least some PrP<sub>Sc</sub> circulates between peri-nuclear and peripheral regions including the plasma membrane via an endocytic recycling pathway.

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) are neurodegenerative disorders characterized by neuronal vacuolization, astrocytosis and accumulation of an abnormal isoform of prion protein (PrP<sub>Sc</sub>) in the central nervous system. PrP<sub>Sc</sub> is considered to be a major component of prions, the causative agent of TSE. PrP<sub>Sc</sub> is a β-sheet-rich structural isomer of a cellular isoform of the prion protein (PrP<sub>C</sub>) and is generated from PrP<sub>C</sub> that is encoded by the Prnp gene of the host cell (Prusiner, 1998). Infectivity of prions is thought to be associated with PrP<sub>Sc</sub> oligomers (Silveira et al., 2005); therefore, generation of PrP<sub>Sc</sub> is a key aspect of prion propagation.

To elucidate the cell biological mechanism of prion propagation, the biosynthesis of PrP<sub>C</sub> and the generation of PrP<sub>Sc</sub> have been investigated using cultured cells susceptible to prion infection. A number of biochemical and cell biological analyses have contributed to elucidation of the intracellular dynamics of PrP<sub>Sc</sub> (Campana et al., 2005). In contrast to the biosynthesis of PrP<sub>C</sub>, the mechanisms by which PrP<sub>Sc</sub> is generated in prion-infected cells are not yet fully understood. One of the obstacles to cell biological analyses of PrP<sub>Sc</sub> is the difficulty in the specific detection of PrP<sub>Sc</sub>. Since PrP<sub>Sc</sub> and PrP<sub>C</sub> have the same primary structure, most of the antibodies against the PrP molecule cannot distinguish PrP<sub>Sc</sub> from PrP<sub>C</sub>. Although some antibodies can specifically immunoprecipitate PrP<sub>Sc</sub> (Horiuchi et al., 2009; Korth et al., 1997; Paramithiotis et al., 2003), these antibodies are not suitable for the detection of PrP<sub>Sc</sub> by immunocytochemical analysis. Taraboulos et al. (1990) reported that pre-treatment of fixed cells with...
guanidinium salts significantly increases the PrP<sup>Sc</sup> signal following immunofluorescence staining. Although the precise reason as to why PrP<sup>Sc</sup>-specific detection is achieved by this pre-treatment remains unclear, this method is now used for the PrP<sup>Sc</sup>-specific detection by immunofluorescence assay (IFA) and immunoelectron microscopy. Earlier studies reported that PrP<sup>Sc</sup> is mainly localized at the plasma membrane, in secondary lysosomes and at a peri-nuclear Golgi region (Fournier et al., 2000; Jeffrey et al., 1992; McKinley et al., 1991; Taraboulos et al., 1990). However, recent immunocytochemical and immunoelectron microscopy studies showed that PrP<sup>Sc</sup> is also localized to early endosomes (Veith et al., 2009), late endosomes/multi-vesicular bodies (Arnold et al., 1995; Pimpinelli et al., 2005) and to endocytic recycling compartment (ERC) at peri-nuclear regions (Godsave et al., 2008; Marijanovic et al., 2009).

In spite of the large contribution of this method on elucidation of the localization of PrP<sup>Sc</sup> in cells, the specificity

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**Fig. 1.** Reactivity of mAbs to PrP in N2a-3 or ScN2a-3-22L cells with or without GdnSCN pre-treatment in IFA. N2a-3 cells and ScN2a-3-22L cells grown in chamber slides were fixed with 4% paraformaldehyde and then permeabilized with 0.1% saponin. The cells were treated with 5 M GdnSCN (b, d, f, h, i, l, n, p, r and t) or were left untreated (a, c, e, g, i, k, m, o, q and s), prior to antibody reaction. The anti-PrP mAbs 31C6 (e–h), 44B1 (i–l), 149 (m–p) and 132 (q–t) were used. The mAb P2-284 was used as a negative control (a–d). Cell nuclei were counterstained with DAPI. Merged images of PrP signals (green) and nuclei (blue) are shown. Nearly 100% ScN2a-3-22L cells were positive for PrP<sup>Sc</sup> in IFA using mAb 132.
of PrPSc detection by this method should be carefully re-evaluated. In fact, pre-treatment with guanidine hydrochloride (GdnHCl) does not prevent detection of PrPSc in uninfected cells (Taraboulos et al., 1990; Veith et al., 2009). In order to distinguish PrPSc from PrPC by IFA, the detector gain or the exposure time needs to be adjusted to a level at which PrPSc signals are below the detection limit (Marijanovic et al., 2009; Veith et al., 2009). Alternatively, prior to pre-treatment with denaturants, proteinase K (PK) treatment is required to completely abolish PrPC signals (Taraboulos et al., 1990; Veith et al., 2009). However, PK treatment affects cell architecture, which makes it difficult to finely analyse localization (unpublished observation). Resolution of these technical limitations will improve the quality of PrPSc detection in prion-infected cells. Taking these background studies into account, we first investigated the utility of various anti-PrP antibodies that recognize different epitopes on the PrP molecule for PrPSc-specific detection using IFA. We found that the mAb 132, which recognizes linear epitope consisting of mouse PrP aa 119–127 (Kim et al., 2004b), the region adjacent to a hydrophobic amino acid sequence of PrP, AGAAAAGA, was the most suitable for this purpose. We therefore extensively analysed the localization of PrPSc in prion-infected cells using mAb 132. Our results suggest that PrPSc is trafficked through a cellular compartment at peri-nuclear regions along with the membrane trafficking machinery of the cells.

RESULTS

Specific detection of PrPSc in prion-infected cells by IFA

Pre-treatment of cells with denaturants such as guanidine thiocyanate (GdnSCN) pre-treatment were stained with mAbs 31C6 or 44B1 that react strongly with PrPSc-specific detection using IFA. We found that the mAb 132, which recognizes linear epitope consisting of mouse PrP aa 119–127 (Kim et al., 2004b), the region adjacent to a hydrophobic amino acid sequence of PrP, AGAAAAGA, was the most suitable for this purpose. We therefore extensively analysed the localization of PrPSc in prion-infected cells using mAb 132. Our results suggest that PrPSc is trafficked through a cellular compartment at peri-nuclear regions along with the membrane trafficking machinery of the cells.

![Fig. 2. PrPSc-specific detection in prion-infected cells using mAb 132. Non-infected N2a-3 and GT1-7 cells as well as ScN2a-3-22L, ScN2a-3-Ch and ScGT1-7-22L cells, were subjected to PrPSc-specific staining using mAb 132. The images in the upper panel show the PrPSc signals (green) and those in the bottom panel are differential interference contrast (DIC) images merged with PrP (mAb 132, green) and nuclear (DAPI, blue) fluorescent images. Bars, 10 μm.](image-url)
Fig. 3. Intracellular localization of PrPSc. ScN2a-3-22L cells were stained with the mAb 132 (PrPSc-specific staining, green) and antibodies against the organelle marker molecules indicated on the left (red). Images in the rightmost column show high magnification images of the boxed regions in the corresponding merged images. The arrows indicate representative examples of co-localization of PrPSc with the corresponding organelle markers (appear yellow), while the arrowheads indicate PrPSc that does not co-localize with the corresponding organelle markers (appear green). Bars, 10 μm.
We next tested mAbs 149 and 132, which display little reactivity with PrP<sup>S</sup>C on the surface of N2a cells by flow cytometric analysis (Kim et al., 2004a). mAb 149 detected faint PrP signals in both N2a-3 and ScN2a-3-22L cells without GdnSCN pre-treatment (Fig. 1m, o). However, GdnSCN pre-treatment abolished the faint PrP<sup>S</sup>C signals detected in N2a-3 cells, but enhanced strong granular signals at peri-nuclear regions in ScN2a-3-22L cells (Fig. 1n, p). mAb 132 staining resulted in intense PrP signals at peri-nuclear regions in ScN2a-3-22L cells pre-treated with GdnSCN (Fig. 1t). Remarkably, this mAb detected little PrP<sup>C</sup>G in N2a-3 cells, regardless of the presence or absence of GdnSCN pre-treatment (Fig. 1q, r). Moreover, PrP signals in ScN2a-3-22L cells without GdnSCN pre-treatment appeared to be below the detection limit (Fig. 1s). We also verified the utility of mAb 132 for PrP<sup>S</sup>C-specific detection in different prion strains or cell lines (Fig. 2). Similar to ScN2a-3-22L cells, characteristic granular signals were detected in N2a-3 cells infected with ScN2a-3-22L cells infected with the Chandler strain (ScN2a-3-Ch) and in GT1-7 cells persistently infected with the 22L strain (ScGT1-7-22L), but fluorescent PrP<sup>C</sup>S signals in uninfected cells remained at background level. Since hardly any PrP<sup>C</sup>S signal was detected in uninfected cells even after GdnSCN pre-treatment, mAb 132 is believed to enable more precise PrP<sup>S</sup>C-specific detection in IFA.

**Intracellular localization of PrP<sup>S</sup>C**

Since mAb 132 enables reliable PrP<sup>S</sup>C-specific detection, we used this mAb to analyse the intracellular localization of PrP<sup>S</sup>C in ScN2a-3-22L cells. Lamp1, a marker of late endosomes and/or lysosomes, has been reported to co-localize with PrP<sup>S</sup>C in prion-infected cells (Pimpinelli et al., 2005). As expected, some PrP<sup>S</sup>C did co-localize with Lamp1 at peri-nuclear and peripheral regions of the cell (Fig. 3, arrows). However, PrP<sup>S</sup>C that was detected in a region extremely close to the nucleus did not co-localize with Lamp1 (Fig. 3, arrowheads).

We further analysed the co-localization of PrP<sup>S</sup>C with Rab GTPases that are known to be present in distinct organelles (Stenmark, 2009; Grant & Donaldson, 2009). Peri-nuclear PrP<sup>S</sup>C partially co-localized with Rab4a, a marker of early endosomes including rapid endocytic recycling endosomes; Rab5a, a marker of early endosomes; Rab7, a marker of late endosomes; Rab9, a marker of late endosomes involved in retrograde transport to the trans-Golgi network; and Rab11a, a marker of ERC (Fig. 3, arrows). In contrast to the co-localization of PrP<sup>S</sup>C with endosomal and lysosomal markers, only a small proportion of PrP<sup>S</sup>C co-localized with Tgn38, a marker of the trans-Golgi network. However, peri-nuclear PrP<sup>S</sup>C did not appear to co-localize with gigantin, a marker of the cis-medial-Golgi (Fig. 3).

Flotillin-1 is a protein that is associated with lipid rafts and is known to be present at the plasma membrane, the trans-Golgi network and in endosomes, lysosomes and lipid droplets (Browman et al., 2007). In contrast to the partial co-localization of PrP<sup>S</sup>C with the endosomal and lysosomal markers described above, a large proportion of PrP<sup>S</sup>C appeared to co-localize with flotillin-1 (Fig. 3).

**PrP<sup>S</sup>C is localized to ERC in the intracellular transport pathway of transferrin (Tfn), Shiga toxin B subunit (StxB) and Cholera toxin B subunit (CtxB)**

Recent ultrastructural and immunofluorescence studies have revealed that PrP<sup>S</sup>C is localized in endocytic compartments (Godsave et al., 2008; Marijanovic et al., 2009; Pimpinelli et al., 2005; Veith et al., 2009). Since PrP<sup>S</sup>C is often detected at the peri-nuclear regions very close to but not in the Golgi apparatus, of particular interest is the ERC that is located near the microtubule organizing centre and the Golgi apparatus that is involved in the recycling of membrane lipids and proteins (Grant & Donaldson, 2009). Therefore, to more precisely analyse juxtanuclear PrP<sup>S</sup>C localization, we used Tfn as a marker for the ERC. Tfn
binds the Tfn receptor that is internalized from the cell surface via clathrin-coated pits, is transported to early endosomes, and is then recycled back to the plasma membrane via the ERC (Maxfield & McGraw, 2004). When the cells were loaded with Alexa-Flour-555-conjugated Tfn, Tfn was detected at the peri-nuclear regions 15 min after the initiation of uptake (time 0) and Tfn had almost disappeared 30 min after the removal of Tfn from the medium (Fig. 4a). Some of the juxtanuclear PrPSc did co-localize with internalized Tfn at peri-nuclear regions 15 min after Tfn loading, suggesting that the ERC is one of the compartments to which PrPSc localizes.

Flotillin-1 is reported to be involved in clathrin-independent endocytosis of StxB and CtxB (Glebov et al., 2006; Lin & Gutman, 2010). StxB and CtxB are known to bind globothriaosylceramide and GM1 ganglioside, respectively, at cell surface and are transported from the plasma membrane to the trans-Golgi network via early endosomes and the ERC (Lieu & Gleeson, 2010; Nichols et al., 2001). Since PrPSc clearly co-localized with flotillin-1 (Fig. 3), we further analysed the localization of PrPSc with StxB and CtxB. When cells were loaded with StxB at 4 °C and StxB was then internalized by transfer of the cells to 37 °C, StxB partly co-localized with PrPSc at the peri-nuclear region 10 min after initiation of internalization (Fig. 4b, arrow). However, co-localization of PrPSc with StxB was not so clear after further 10 min incubation (Fig. 4b, arrowhead). Similarly, co-localization of PrPSc with internalized CtxB was observed at the peri-nuclear regions 15 and 30 min after initiation of internalization, but co-localization was weaker after 60 min (Fig. 4c). At 15–30 min after initiation of internalization, CtxB at the peri-nuclear regions showed strong co-localization with Rab11a, but after 60 min, it co-localized more strongly with Tgn38. This result suggested that CtxB present at the peri-nuclear regions was mainly in the ERC at 15–30 min, and in the trans-Golgi network at 60 min after initiation of internalization (Fig. 5, 37 °C). During subsequent 1 h incubation, PrPSc signals at the peri-nuclear regions appeared to decrease within 30 min of incubation (Fig. 6a, 37 °C/30 min). Interestingly, after an additional 30 min incubation at 37 °C, some of the PrPSc reappeared at the peri-nuclear regions including the plasma membrane, although some PrPSc was still detected at the peri-nuclear regions (Fig. 6a, 37 °C/60 min, arrowheads). During subsequent 1 h incubation, PrPSc signals at the peripheral regions decreased again, and thereafter, signals of PrPSc at the peri-nuclear regions appeared to revert to the steady-state level (Fig. 6a, 37 °C/120 min). Little PrPSc was detected in N2a-3 cells throughout the experimental period (Fig. 6b), which means that both the peri-nuclear and the peripheral fluorescent signals represent PrPSc. In spite of the changes in the intracellular distribution of PrPSc, the amount of PrP-res, a representative of a protease-resistant form of PrPSc, was unchanged over the experimental period (Fig. 7). These results suggest that the dynamic changes in the distribution of PrPSc were not due to major differences in synthesis or degradation of PrPSc at specific regions of the cell, but were primarily due to the intracellular trafficking of PrPSc through the peri-nuclear regions.

**PrPSc dynamically cycles between peripheral and peri-nuclear regions in ScN2a-3-22L**

The presence of PrPSc in various endocytic compartments, especially in the ERC through which Tfn, StxB and CtxB are transported, suggested that PrPSc is transported intracellularly by the membrane trafficking machinery of the cell, rather than being a permanent resident of these organelles. To address the association of PrPSc with the intracellular membrane trafficking machinery, we analysed the localization of PrPSc in ScN2a-3-22L cells that were incubated at 20 °C. Incubating cells at a low temperature (≤ 20 °C) impairs the transport of Tfn or StxB from the plasma membrane to peri-nuclear regions (Mallard et al., 1998; Ren et al., 1998; Sipe et al., 1991). We firstly confirmed that incubation of ScN2a-3-22L cells at 20 °C slowed down the transport of CtxB (Fig. 5) and StxB (data not shown). Following incubation at 37 °C, CtxB was concentrated at the peri-nuclear regions and co-localized with Rab11a within 15–30 min and with Tgn38 within 60 min, after initiation of its uptake. In contrast, CtxB did not co-localize with either Rab11a or Tga38 after 30 min of incubation at 20 °C, but co-localization with Rab11a could be observed 60 min after the start of incubation. In addition, some CtxB still remained at the plasma membrane 60 min after the incubation, indicating that the transport of CtxB from the plasma membrane to peri-nuclear regions had been slowed down. Under this condition, most of the PrPSc signals disappeared from the peri-nuclear regions and appeared at peripheral regions of the cells, including at the plasma membrane, within 1 h (Fig. 6a, 20 °C/1 h, arrowheads). After subsequent incubation for another hour, some of the PrPSc seemed to have redistributed to the peri-nuclear regions (Fig. 6a, 20 °C/2 h), following which, PrPSc gradually disappeared again from the peri-nuclear regions over the next 10 h of incubation. Indeed, following this incubation, the majority of PrPSc redistributed to the peripheral regions of the cells (Fig. 6a, 20 °C/12 h).

When ScN2a-3-22L cells that had been incubated at 20 °C for 12 h were transferred to 37 °C, peri-nuclear PrPSc signals appeared to increase and PrPSc signals at the peripheral regions appeared to decrease within 30 min of incubation (Fig. 6a, 37 °C/30 min). Interestingly, after an additional 30 min incubation at 37 °C, some of the PrPSc reappeared at the peripheral regions including the plasma membrane, although some PrPSc was still detected at the peri-nuclear regions (Fig. 6a, 37 °C/60 min, arrowheads). During subsequent 1 h incubation, PrPSc signals at the peripheral regions decreased again, and thereafter, signals of PrPSc at the peri-nuclear regions appeared to revert to the steady-state level (Fig. 6a, 37 °C/120 min). Little PrPSc was detected in N2a-3 cells throughout the experimental period (Fig. 6b), which means that both the peri-nuclear and the peripheral fluorescent signals represent PrPSc. In spite of the changes in the intracellular distribution of PrPSc, the amount of PrP-res, a representative of a protease-resistant form of PrPSc, was unchanged over the experimental period (Fig. 7). These results suggest that the dynamic changes in the distribution of PrPSc were not due to major differences in synthesis or degradation of PrPSc at specific regions of the cell, but were primarily due to the intracellular trafficking of PrPSc through the peri-nuclear regions.

**DISCUSSION**

Analysis of the intracellular localization of PrPSc is important for the understanding of the cellular mechanism of prion propagation. Taraboulos et al. (1990) showed that pre-treatment of prion-infected cells with GdnHCl enables PrPSc-specific detection using IFA. Although the mechanism of
PrPSc-specific detection by pre-treatment of cells with chaotrophic agents was unclear; this method has been used for the detection of PrPSc in cells and tissues (Marijanovic et al., 2009; Pimpinelli et al., 2005; Taraboulos et al., 1990; Veith et al., 2009). Treatment with denaturant facilitates exposure of cryptic epitopes of PrPSc (Taraboulos et al., 1990). However, since GdnHCl treatment does not remove PrPSc from uninfected cells (Taraboulos et al., 1990; Veith et al., 2009; this study), detection of PrPSc signals would be expected because most of the antibodies against PrP molecules react not only with denatured PrPSc but also with denatured PrPC. If signals from the denatured PrPSc are strong enough to distinguish them from PrPSc, then PrPSc-specific detection can be achieved by setting the threshold level just above the level of PrPSc signals by manipulation of the gain of detector, changing exposure time and so on. However, such adjustment will miss subtle details of the PrPSc signals. Therefore, further improvement of PrPSc-specific detection is still required for detailed characterization of the intracellular localization of PrPSc. To improve the specificity of PrPSc detection, we used mAb 132, which recognizes the epitope consisting of mouse PrP aa 119–127, adjacent to the hydrophobic amino acid sequence, AGAAAAGA (Gasset et al., 1992). Since mAb 132 showed little reactivity with native PrPC on the cell surface, the epitope for mAb 132 is thought to be buried inside the PrPC molecule. Denaturation with GdnSCN was expected to expose the epitope for mAb 132 of PrPSc, but in fact, mAb 132 showed little reactivity with PrPC in uninfected cells even after treatment with GdnSCN (Fig. 1). A reduction in PrPSc

Fig. 5. Localization of CtxB with marker molecules. ScN2a-3-22L cells were incubated with medium containing Alexa-Fluor-488-conjugated CtxB at 4°C for 30 min and the medium was then replaced with CtxB-free fresh medium. The cells were then incubated at 37°C (top panel) or 20°C (bottom panel) for up to 60 min. The cells were fixed at each time point and stained with anti-Rab11a or anti-Tgn38 rabbit polyclonal antibodies. Merged images of Rab11a or Tgn38 (green) with CtxB (red) and nuclei (blue) are shown. Bars, 10 μm.
Fig. 6. Changes in the localization of PrPSc during incubation at 20 °C and subsequent incubation at 37 °C. (a) Temperature-dependent localization of PrPSc in ScN2a-3-22L cells. ScN2a-3-22L cells were grown on a chamber slide at 37 °C. The cells were then incubated at 20 °C for the indicated periods for up to 12 h. After 12 h incubation at 20 °C, the cells were incubated at 37 °C for up to 120 min as indicated. The cells were then fixed and subjected to PrPSc-specific staining using mAb 132. The upper panels show merged images of PrPSc (green) and nuclei (blue). The bottom panels show high magnification images of the boxed regions in the corresponding upper panels that were merged with a DIC image. The arrowheads point to representative examples of PrPSc signals at the cell surface. Bar, 10 μm. (b) Specificity of PrPSc staining. N2a-3 or ScN2a-3-22L cells cultured on a chamber slide at 37 °C were subsequently incubated at 20 °C for 12 h and then incubated at 37 °C for 60 min. These cells were fixed and subjected to PrPSc-specific staining using the mAb132. Bar, 10 μm.

Intracellular localization of PrPSc

Fig. 7. Amount of PrP-res. ScN2a-3-22L cells cultured on 60 mm dishes at 37 °C were transferred to 20 °C and incubated for 1–12 h. After 12 h of incubation, the cells were incubated again at 37 °C for 15–120 min. The cells were lysed at the indicated time points and were subjected to immunoblotting for PrP-res detection. Sample volume equivalent to 4×10⁶ cells were loaded on each lane. A representative immunoblot image is shown on the top and the graph at the bottom shows the level of PrP-res relative to its level at time 0. The means and SD of four independent experiments are depicted.

labelling is an important factor for PrPSc-specific detection (Dron et al., 2009). Indeed, this property of the mAb 132 allowed us to perform more precise PrPSc detection using IFA because the lack of, or trace PrPSc signal means that specific manipulation of the threshold setting during data acquisition can be minimized. Since mAb 132 reacts with denatured PrPSc and PrPSc in immunoblotting (Kim et al., 2004b), the reason why mAb 132 cannot detect PrPSc, but can detect PrPSc, in cells pre-treated with GdnSCN remains unclear. However, one possible explanation is that the epitope for mAb 132 on PrPSc may not be exposed by the relatively short denaturation period (10 min exposure to GdnSCN). Alternatively, it is possible that once the epitope for mAb 132 on PrPSc has been exposed by denaturation with GdnSCN, PrPSc may quickly refold into a conformation that prevents access of mAb 132 to the epitope after the removal of GdnSCN. In contrast, PrPSc was detected in prion-infected cells after denaturation with GdnSCN (Fig. 1), indicating that the epitope-containing region of PrPSc molecules, unlike that of PrPSc, remained antibody-accessible. PrPSc has been reported to refold into a PrPSc-like form after strong denaturation with GdnHCl (Callahan et al., 2001; Kocisko et al., 1996). Given that short-term exposure of GdnSCN will not completely denature PrPSc, and that PrPSc exists as an oligomer, intermolecular interaction among neighbouring PrP molecules may disturb the refolding of denatured PrPSc and as a consequence, the epitope for mAb 132 on PrPSc molecule may remain accessible to the antibody.

In agreement with previous reports (Marijanovic et al., 2009; Pimpinelli et al., 2005; Taraboulos et al., 1990; Veith et al., 2009), we confirmed the widespread intracellular distribution of PrPSc throughout endocytic compartments in prion-infected cells (Fig. 3). PrPSc was not only present in early and late endosomes/lysosomes but also in cellular compartments at the peri-nuclear regions, at least some of which are thought to be the ERC, through which exogenously derived Tfn, StxB and CtxB are transported during their intracellular trafficking (Fig. 4). In this study, we observed the extensive co-localization of PrPSc with flotillin-1 in ScN2a-3-22L cells (Fig. 3). This result is inconsistent with the report by Pimpinelli et al. (2005), in which PrPSc in the Chandler strain-infected N2a cells was not co-localized well with flotillin-1. It remains to be elucidated how differences in prion strains and cell types influence the intracellular distribution of PrPSc.

Recent immunofluorescent and electron microscopy studies have also proposed the presence of PrPSc at the ERC (Godsave et al., 2008; Marijanovic et al., 2009). The ERC is a membranous tubular compartment in the vicinity of the nucleus and is defined by the presence of Rab11a and/or Tfn-bound Tfn receptor (Grant & Donaldson, 2009; Murphy et al., 2005). Some internalized plasma membrane proteins such as Tfn receptor, the LDL receptor and GPI-anchored proteins including PrPSc are transported from the early endosomes to the ERC and are then sorted to their target sites including the plasma membrane (Magalhães et al., 2002; Mayor & Riezman 2004; Morris et al., 2006;
Prado et al., 2004). Many proteins that cycle between the intracellular compartments and the cell surface are known to accumulate in the ERC (Maxfield & McGraw, 2004). Thus, the presence of PrPSc in prion-infected cells is intracellularly transported with the membrane trafficking machinery that is associated with endocytic recycling. Although time-lapse imaging of trafficking PrPSc in cells persistently infected with prions is technologically impossible at the moment, the kinetic analysis of PrPSc distribution in the cells shown in this study provided an interesting insight into the intracellular trafficking of PrPSc (Fig. 6). PrPSc granules at the peripheral regions including those at the plasma membrane appeared to increase during incubation at 20 °C, whereas peri-nuclear PrPSc granules gradually decreased under this condition. Conversely, peripheral PrPSc granules appeared to decrease as PrPSc granules accumulated at the peri-nuclear regions once the cells were transferred to 37 °C. This drastic alteration in the distribution of PrPSc during sequential incubation at 20 and 37 °C suggests that PrPSc is dynamically transported through compartments that exist at the peri-nuclear regions, at least some of which are thought to be the ERC, by the membrane trafficking machinery of the cell. Combined with the fact that PrPSc is present at the ERC as described above, it is conceivable that at least some of the PrPSc cycles between peri-nuclear and peripheral regions including the plasma membrane through the ERC, via the endocytic recycling pathway.

Marijanovic et al. (2009) reported that impairment of the transport from early endosomes to the ERC by overexpression of Rab22a reduced the level of PrPSc, but that impairment of transport from the ERC to the plasma membrane using a dominant-negative mutant of Rab11a increased the level of PrPSc in GT1 cells infected with prions. These findings suggest that the ERC is possibly one of the sites where PrPSc formation takes place. Although multiple trafficking pathways may be involved in the intracellular transport of PrPSc as suggested by the widespread distribution of PrPSc in prion-infected cells, cycling of PrPSc between the peri-nuclear and peripheral regions including the plasma membrane through the ERC, rather than accumulation of PrPSc at specific compartments, may provide a significant advantage for the generation of PrPSc. For instance, such cycling may provide increased opportunities for PrPSc to enter a site for conversion, which could then act as a seed for the conversion of PrPSc into PrPSc.

There are a few studies which report that PrPSc was detected in late endosomes or early endosomes/ERC in the brains of prion-infected animals (Arnold et al., 1995; Godsave et al., 2008). However, intracellular localization of PrPSc in brains of prion-infected animals is largely unknown. We have confirmed that mAb 132 is also applicable to the PrPSc-specific detection in brain section of prion-infected animals, and now experiments are under way to extensively analyse the cellular compartments where prions present in the brain of mice infected with prions.

**METHODS**

**Antibodies, reagents and chemicals.** Mouse mAbs, 31C6, 44B1, 149 and 132 were used for the detection of PrP, mAbs 31C6, 149 and 132 recognize linear epitopes consisting of mouse PrP aa 143–149, 147–151 and 119–127, respectively, whereas mAb 44B1 recognizes a discontinuous epitope consisting of aa 155–231 (Kim et al., 2004b). Following antibodies were used for IFA: anti-Łamp1 rat mAb (1D4B; Beckman Coulter); rabbit polyclonal antibodies: anti-Tgn38 (ab16599; Abcam), anti-gitatin (PRR-114C; Covance), anti-flotillin-1 (F1180; Sigma), anti-Rab5 (#2143), anti-Rab7 (#2094) and anti-Rab11a (#3539) (all three from Cell Signaling Technology), anti-Rab4a (10347-1-AP) and anti-Rab9 (11420-1-AP) (Proteintech Group Inc.). An affinity purified anti-StxB rabbit polyclonal antibody was prepared from polyclonal antisera raised by immunizing a rabbit with purified Stxb1-H and Stxb2-H (Shimizu et al., 2007). Alexa-Fluor-488- or 546-conjugated goat F(ab')2 fragment anti-mouse IgG, Alexa-Fluor-555-conjugated goat F(ab')2 fragment anti-rabbit IgG and Alexa-Fluor-555-conjugated goat IgG anti-rat IgG (Invitrogen) were used as secondary antibodies for IFA.

**Cell culture.** Subclones of mouse neuroblastoma cell line Neuro2a, N2a-3 (Uryu et al., 2007), and of the hypothalamic neuronal cell line GT1, GT1-1 (Schatzl et al., 1997), were used. As prion-infected cells, ScN2a-3-Ch (Uryu et al., 2007), ScN2a-22L (Nakamitsu et al., 2010) and ScGT1-7-22L were used. These prion-infected cells maintain PrPSc without significant loss of PrPSc more than 50 passages. However, we usually used these cells of passage history between 10 and 30 times. N2a-derived cells were passaged at a 1:10 dilution ratio every 3–4 days and were cultured in Dulbecco’s modified Eagle’s medium (DMEM; ICN Biomedicals) containing 10 % PBS (Gibco), MEM non-essential amino acids (NEAA; Gibco), penicillin (100 U ml⁻¹) and streptomycin (100 µg ml⁻¹) at 37 °C in 5 % CO₂ atmosphere. The GT1-7 cells were passaged at a 1:5 dilution ratio every 7 days and were cultured in DMEM containing 5 % FBS, 5 % horse serum (Gibco) and penicillin/streptomycin.

**IFIA.** Cells grown on a Lab-Tek II CC2 eight-well chamber slide (Nunc) were fixed with pre-warmed PBS containing 0.1 M glycine in PBS for 10 min. After removal of the fixation solution, the remaining cells were incubated with primary antibodies in PBS containing 0.5 % FBS at 4 °C overnight. Anti-PrP mAbs were used at a concentration of 1 µg ml⁻¹. A mAb P2-284 (1 µg ml⁻¹) against feline panleukopenia virus was used as a negative-control antibody (Horiiuchi et al., 1997). After washing five times with PBS, the cells were incubated with Alexa-Fluor-conjugated secondary antibodies (1:1000) at RT for 90 min. For counterstaining of cell nuclei, the cells were incubated for 30 min with 5 µg DAPI ml⁻¹ (Invitrogen) in PBS at RT. The samples were then mounted with ProLong Gold antifade reagent (Invitrogen) and covered with coverslips. The samples were examined using a Nikon C1 laser confocal fluorescence microscope (Nikon) or with a Zeiss laser scanning microscope LSM 700 (Zeiss).

**Tfn, CtxB and StxB uptake experiments.** For analysis of Tfn uptake, cells grown on Lab-Tek chamber slide were washed twice with Opti-MEM (Gibco) and incubated with 10 µg Alexa-Fluor-555-conjugated Tfn (Invitrogen) ml⁻¹ in Opti-MEM at 37 °C for 15 min. The cells were then washed three times with pre-warmed PBS and incubated at 37 °C for up to 30 min. For analysis of the uptake of CtxB, cells grown on a Lab-Tek chamber slide were washed with...
Opti-MEM and incubated with 2 μg Alexa-Fluor-488-conjugated CtxB (Invitrogen) ml⁻¹ in Opti-MEM at 4 °C for 30 min. For analysis of StxB uptake (Shimizu et al., 2007), cells grown on a Lab-Tek chamber slide were washed with Opti-MEM and incubated with then replaced with pre-warmed fresh DMEM and the cells were incubated at 37 °C for up to 60 min.

**Immunoblotting of PrP-res.** ScN2a-3-22L cells on 60 mm dishes were washed once with PBS, collected using a cell scraper and the cell number was counted. The collected cells were lysed with lysis buffer (Uryu et al., 2007) at 4 °C for 20 min and cell debris was removed by centrifugation at 2000 g. The protein concentration of the lysates was measured using the DC protein assay kit (Bio-Rad) and was adjusted to 1 mg ml⁻¹. The lysates (300 μl) were digested with 20 μg PK ml⁻¹ at 37 °C for 20 min. The proteolysis was terminated by the addition of Pefabloc (Roche) to 1 mM. The PK-treated lysates were incubated with 0.3 % phosphostigmine acid at RT for 20 min and then centrifuged at 20,000 g for 20 min to precipitate PK-resistant PrP (PrP-res). The pellet was dissolved in SDS sample buffer and boiled for 5 min. A sample volume equivalent to 4 x 10⁶ cells was loaded onto a NuPAGE 12 % Bistris gel (Invitrogen). SDS-PAGE was carried out according to the manufacturer’s instructions (Invitrogen). Subsequent Western transfer and chemiluminescence were carried out as described previously (Shindoh et al., 2009).

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