Characterization of intracellular dynamics of inoculated PrP-res and newly generated PrP^{Sc} during early stage prion infection in Neuro2a cells

Takeshi Yamasaki[1], Gerald S Baron[2], Akio Suzuki[1], Rie Hasebe[1] and Motohiro Horiuchi[1]

[1]Laboratory of Veterinary Hygiene, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo 060-0818, Japan

[2]Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT, USA

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To whom correspondence should be addressed: Motohiro Horiuchi, DVM, Ph.D. Laboratory of Veterinary Hygiene, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo 060-0818 Japan, Phone/Fax: +81-11-706-5293; e-mail: horiuchi@vetmed.hokudai.ac.jp

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Summary

To clarify the cellular mechanisms for the establishment of prion infection, we analyzed the intracellular dynamics of inoculated and newly generated abnormal isoform of prion protein (PrP^{Sc}) in Neuro2a cells. Within 24 h after inoculation, the newly generated PrP^{Sc} was evident at the plasma membrane, in early endosomes, and in late endosomes, but this PrP^{Sc} was barely evident in lysosomes; in contrast, the majority of the inoculated PrP^{Sc} was evident in late endosomes and lysosomes. However, during the subsequent 48 h, the newly generated PrP^{Sc} increased remarkably in early endosomes and recycling endosomes. Overexpression of wild-type and mutant Rab proteins showed that membrane trafficking along not only the endocytic-recycling pathway but also the endo-lysosomal pathway is involved in *de novo* PrP^{Sc} generation. These results suggest that the trafficking of exogenously introduced PrP^{Sc} from the endo-lysosomal pathway to the endocytic-recycling pathway is important for the establishment of prion infection.

Introduction

Prions are causative agents of transmissible spongiform encephalopathies (TSEs), neurodegenerative disorders that are characterized by accumulation of an abnormal isoform of prion protein (PrP^{Sc}) in the central nervous system (CNS). PrP^{Sc} is the only known proteinaceous component of prions, and infectivity of prions is thought to be associated with PrP^{Sc} oligomers (Silveira et al., 2005; Wang et al., 2010). PrP^{Sc} is a conformational isomer of a cellular prion protein (PrP^{C}) and is rich in β-sheet; PrP^{Sc} is generated from PrP^{C}, a protein that is expressed on the surface of host cells (Prusiner, 1998). Conversion of PrP^{C} to PrP^{Sc} is thought to be triggered by direct contact between "seed" PrP^{Sc} and "substrate" PrP^{C}.

The intracellular dynamics of PrP^{Sc} immediately following exposure of the cells to prions have been analyzed to understand the mechanisms by which a prion infection becomes established. Although PrP^{C} is not necessary for the internalization of PrP^{Sc} (Greil et al., 2008; Hijazi et al., 2005; Jen et al., 2010; Magalhaes et al., 2005; Paquet et al., 2007), some candidates such as laminin receptor, heparan sulfate, or low-density lipoprotein receptor-related protein 1 (LRP1) have been reported to act as receptors that mediate internalization of exogenously inoculated PrP^{Sc} (Gauczynski et al., 2006; Horonchik et al., 2005; Jen et al., 2010).

During the early stage after inoculation of PrP^{Sc}, internalized PrP^{Sc} has been reported to be directed to late endosomes/lysosomes via the endo-lysosomal pathway (Jen et al., 2010; Magalhaes et al., 2005). In cells persistently infected with prions, a number of studies have shown that PrP^{Sc} localizes throughout the endocytic compartments — specifically the plasma membrane, early endosomes, recycling endosomes, late endosomes, secondary lysosomes, and the peri-nuclear Golgi region (Marijanovic et al., 2009; McKinley et al., 1991; Pimpinelli

et al., 2005; Taraboulos et al., 1990; Veith et al., 2009; Vey et al., 1996; Yamasaki et al., 2012). Earlier studies suggested that generation of PrP^{Sc} occurs on the cell surface or within the endocytic pathway (Borchelt et al., 1992; Caughey and Raymond, 1991; Taraboulos et al., 1992). Marijanovic *et al.* reported that the endocytic recycling compartment (ERC) may be the site where the conversion of PrP^{C} to PrP^{Sc} occurs (Marijanovic et al., 2009). In a very early stage of prion infection where new PrP^{Sc} formation was measured within minutes after initiation of infection, Goold *et al.* recently reported that the plasma membrane is a primary site of conversion (Goold et al., 2011).

In spite of the efforts described above, the events required for the establishment of prion infection in cells, especially those that occur in the early stage after introduction of the infectious prions, are poorly understood. Simultaneous analysis of inoculated PrP^{Sc} and newly generated PrP^{Sc} in a short period after challenge with prions is important for understanding the early events of prion infection. However, such analysis is limited due to the technical difficulties in the distinction of PrP^{Sc} from PrP^{C} and also in the distinction of newly generated PrP^{Sc} from inoculated PrP^{Sc}. For example, antibody epitope-tagging has been used to specifically detect host cell-derived PrP or input PrP^{Sc} (Greil et al., 2008; Vorberg et al., 2004) but not both. Specific labeling of PrP^{Sc} with anti-PrP antibody via pre-treatment of cells with chaotropic agents such as guanidinium salt has been widely used to detect PrP^{Sc} in cells (Marijanovic et al., 2009; Pimpinelli et al., 2005; Taraboulos et al., 1990). However, this method is limited because weak PrP^{Sc} signals such as newly generated PrP^{Sc} may be lost or obscured because the detector gain or exposure times need to be adjusted to a level at which signals from endogenous PrP^{C} are below the detection limit.

We recently reported that mAb 132, an anti-PrP monoclonal antibody that recognizes an

epitope consisting of amino acids 119-127 of mouse PrP, allowed us to visualize PrP^{Sc} in prion-infected cells by indirect immunofluorescence assay (IFA) without specific manipulation of threshold setting to diminish PrP^{C} signals (Yamasaki et al., 2012). Here, we established a method in which inoculated PrP^{Sc} and newly generated PrP^{Sc} can be distinguished by combining the use of fluorescent-dye-labeled purified proteinase K (PK)-resistant PrP^{Sc} (PrP-res) as inoculum with PrP^{Sc}-specific staining with mAb 132. By using this method, in the present study, we extensively analyzed the fate of the inoculated PrP-res and the appearance of newly generated PrP^{Sc} in Neuro2a cells during the early stages of prion infection.

Results

Purification and labeling of PrP-res

To monitor the intracellular dynamics of PrP-res after inoculation, PrP-res was purified from the brains of mice infected with the 22L or Obihiro scrapie strain and the purified PrP-res was labeled with Alexa Fluor 488 or Alexa Fluor 555 succinimidyl ester. Silver staining and immunoblotting of the labeled and unlabeled preparations showed the expected three major bands ranging from 35 to 20 kDa corresponding to the three PrP glycoforms, demonstrating the purity of the PrP-res fraction (Fig. 1a and b). However, both preparations contained bands less than mol wt 15 kDa that did not react with mAb 31C6 (Fig. 1a). Fluorescence imaging, as well as silver staining and immunoblotting of Alexa Flour 488- or 555-labeled purified PrP-res (Af488-22L-PrP-res or Af555-22L-PrP-res, respectively), revealed that the purity was satisfactory for the analysis of intracellular trafficking of 22L-PrP-res by fluorescent microscopy (Fig. 1b). The size of PrP^{Sc} aggregates influences the

uptake of PrP^{Sc} into cells (Greil et al., 2008; Jen et al., 2010; Magalhaes et al., 2005); therefore, the 22L-PrP-res was extensively sonicated and large aggregates were removed by centrifugation. The PrP-res that largely remained in the supernatant (Fig. 1c) was used as the inoculum for N2a-3 cells, a Neuro2a clone which is susceptible to prion infection (Uryu et al., 2007). As was the case in previous studies (Jen et al., 2010; Magalhaes et al., 2005), the Af555-22L-PrP-res that was internalized by cells was dynamically moving throughout the cells at 6 h after inoculation (Supplementary video 1). Most of the Af555-22L-PrP-res particles were co-localized with PrP^{Sc} signals by immuno-staining with mAb 132 (Fig. 1d), which demonstrated the utility of detection of Alexa Fluor 555 signals for monitoring the trafficking of inoculated PrP-res by fluorescence microscopy.

Intracellular localization of inoculated PrP-res

To analyze the intracellular trafficking of the inoculated PrP-res, particularly in the very early stages after internalization, trafficking of the Af555-22L-PrP-res was monitored in cells that transiently express enhanced green fluorescent protein （EGFP） -tagged wild-type Rab4a, 5a, 7, 9, 11a, or 22a as markers of distinct endocytic compartments as follows: Rab4a, early endosomes including rapid endocytic recycling endosomes; Rab5a, early endosomes; Rab7, late endosomes; Rab9, late endosomes involved in retrograde transport to the *trans*-Golgi network (TGN); Rab11a, recycling endosomes; Rab22a, early endosomes involved in transport to recycling endosomes and TGN (Stenmark, 2009). To reduce the possibility of observing ectopically targeted Rab GTPase-EGFP fusion proteins, cells showing relatively weak EGFP signals were selected for these observations. Within 2 h after the initiation of incubation with Af555-22L-PrP-res, we observed particles of Af555-22L-PrP-res

that were incorporated into the EGFP-positive vesicles and thereafter, PrP-res particles moved together with the EGFP-positive vesicles throughout the cells. These dynamics were observed in cells that expressed any type of Rab GTPase-EGFP fusion protein (Fig. 2, arrows). These observations indicated that exogenously introduced PrP-res was most likely transported throughout endocytic compartments within a short period after the internalization.

Transferrin (Tfn) binds to the Tfn receptor, which is internalized from the cell surface by clathrin-coated pits, transported to early endosomes and then recycled back to the plasma membrane via the ERC (Maxfield and McGraw, 2004). In contrast, low-density lipoprotein (LDL) binds to the LDL receptor, which is also internalized from cell surface via clathrin-coated pits; however, after dissociation from the LDL receptor in early endosomes, LDL is transported to late endosomes for degradation (Ikonen, 2008). Herein we define the pathway by which the Tfn receptor is recycled between the plasma membrane and the ERC as "endocytic-recycling pathway" and the pathway by which LDL is directed to late endosomes or lysosomes for degradation as "endo-lysosomal pathway". To analyze the trafficking of PrP-res immediately after internalization, Af555-22L-PrP-res and Alexa Fluor 488-conjugated Tfn (Af488-Tfn) or Alexa Fluor 488-conjugated LDL (Af488-LDL) were inoculated simultaneously into N2a-3 cells. Time-lapse imaging showed that within 2 h after the initiation of incubation with PrP-res, a particle of Af555-22L-PrP-res on the cell surface was internalized and merged with Af488-Tfn signals (Fig. 3a, between 1 min 40 s and 4 min 30 s) during transport to a peri-nuclear region, and thereafter, Af555-PrP-res and Af488-Tfn moved together back to a peripheral region of the cell (*e.g.*, 10 min 20 s). We also observed the internalization of an Af555-22L-PrP-res particle from the cell surface that subsequently merged with an Af488-LDL-positive vesicle (Fig. 3b, between 7 min 50 sec and 9 min 5 s),

and thereafter, Af555-PrP-res and Af488-LDL moved together (*e.g.*, 10 min 5 s).

To clarify the trafficking pathway of the inoculated PrP-res, cells incubated with Af555-22L-PrP-res for 6 h were subsequently cultured for up to 30 h. Six hours after the incubation with PrP-res, some of Af555-22L-PrP-res signals co-localized with Af488-Tfn signal not at the center of the cluster of Af488-Tfn signal, but at a region around the cluster (Fig. 3c, Tfn, 0 h). On the other hand, a large portion of the Af555-22L-PrP-res appeared to co-localize with Af488-LDL throughout the cells (Fig. 3c, LDL, 0h). Quantitative analysis of the co-localization revealed that Af555-22L-PrP-res co-localized more with Af488-LDL (53%) than AF488-Tfn (26%). The co-localization of Af555-22L-PrP-res with Af488-LDL was still obvious (49%) 30 h after the inoculation, whereas, the co-localization of Af555-22L-PrP-res with Af488-Tfn had apparently decreased (4%) (Fig. 3c, LDL and Tfn, 30 h). These results suggested that internalized PrP-res entered both the endocytic-recycling pathway and the endo-lysosomal pathway immediately following internalization, but that a large portion of the inoculated PrP-res was eventually directed to the endo-lysosomal pathway.

The kinetics of inoculated PrP-res metabolism and generation of PrP^{Sc}

To determine the fate of the inoculated PrP-res and detect *de novo* generation of PrP^{Sc}, the PrP-res level in cells inoculated with Af488-22L-PrP-res was analyzed with immunoblotting. The level of inoculated Af488-22L-PrP-res, which was detected with an anti-Alexa Fluor 488 antibody, was drastically decreased within 24 h post inoculation (hpi) (Fig. 4a). In contrast, the total PrP-res level, which was detected with an anti-PrP antibody reactive to both the inoculated PrP-res and newly generated PrP^{Sc}, was unchanged at 24 hpi but increased

thereafter (Fig. 4a). The decrease in the inoculated PrP-res was also consistent with the decrease in PrP-res signal observed by live-cell imaging of Af555-22L-PrP-res over the same time period (Fig. 4b). The increase of the mono-glycosylated PrP-res on immunoblots of total PrP-res at 24 hpi suggested that the *de novo* generation of PrP^{Sc} takes place within 24 hpi (Fig. 4a, arrowhead). Therefore, we analyzed the inoculated PrP-res and newly generated PrP^{Sc} simultaneously in individual cells by IFA. We used Af555-22L-PrP-res in combination with mAb 132-mediated specific detection of PrP^{Sc} to distinguish *de novo* PrP^{Sc}, which could be detected only with mAb 132 (Fig. 4c, arrows), from exogenous PrP-res, which was labeled both with Alexa Fluor 555 and mAb 132 (Fig. 4c, arrowheads). Signals from newly generated PrP^{Sc} became detectable at 24 hpi, especially at a peri-nuclear region of the cells (arrows), and the number of newly generated PrP^{Sc} granules and their fluorescent intensities increased thereafter. In contrast to the increase of newly generated PrP^{Sc}, the inoculated PrP-res decreased with time (Fig. 4c, arrowheads).

Intracellular localization of inoculated PrP-res and newly generated PrP^{Sc}

To identify the site of *de novo* generation of PrP^{Sc}, we next analyzed the intracellular localization of inoculated Af555-22L-PrP-res and newly generated PrP^{Sc} with markers for endocytic compartments. At 24 hpi, some fluorescent signals from the newly generated PrP^{Sc} co-localized well with EEA1 (early endosomes) and Rab7 (late endosomes), but were poorly co-localized with Rab11a (recycling endosomes) or cathepsin D (lysosomes) (Fig. 5). Furthermore, faint signals from newly generated PrP^{Sc} could be detected at the cell surface at 24 hpi. The intensities of these PrP^{Sc} signals increased with time after inoculation (Fig. 5, from 24 to 72 hpi). In order to clarify the kinetics of the distribution of the inoculated PrP-res

and newly generated PrP^{Sc}, we quantitatively analyzed co-localization ratios of the inoculated PrP-res and newly generated PrP^{Sc} co-localized with markers (Fig. 6). Consistent with the data from the live-cell imaging (Fig. 3), a large proportion of the inoculated PrP-res localized to late endosomes (40%) while a smaller portion was detected at the cell surface (22%) at 0 hpi. The exogenous PrP-res at the cell surface almost disappeared during the subsequent 48 h and the remaining PrP-res localized to late endosomes (from 16% to 14%) and lysosomes (from 11% to 9%). In contrast, at 24 hpi newly generated PrP^{Sc} localized mainly at the cell surface (27%), in early endosomes (15%), and late endosomes (30%), but a minor portion localized to lysosomes (3%). Interestingly, the proportion of newly generated PrP^{Sc} localized to early endosomes or recycling endosomes increased (from 15% to 30% and from 5% to 33%, respectively); in contrast, the amount of PrP^{Sc} at the cell surface decreased from 27% to 3% with time after inoculation. These data suggested that even though newly generated PrP^{Sc} was detected in late endosomes and lysosomes at 24 hpi, newly generated PrP^{Sc} remarkably appeared in the intracellular organelles on the endocytic-recycling pathway thereafter.

Influence of the impairment of intracellular transport on de novo generation of PrP^{Sc}

To determine which pathway of intracellular transport is involved in *de novo* generation of PrP^{Sc}, we analyzed the levels of newly generated PrP^{Sc} in cells in which trafficking between the endocytic compartments was selectively impaired by the overexpression of wild-type or dominant-negative mutants of Rab GTPase proteins (Fig. 7). The impairment of the endocytic-recycling pathway by overexpression of wild-type Rab22a or a dominant-negative mutant of Rab11a, which are known to affect the transport from early endosomes to recycling

endosomes (Magadan et al., 2006) or the transport from recycling endosomes to plasma membrane (Ren et al., 1998), respectively, reduced the amount of *de novo* generated PrP^{Sc} at 48 hpi by 62% or 69% of the control (Fig. 7). Additionally, the impairment of the endo-lysosomal pathway by the overexpression of a dominant-negative mutant of Rab7, which is known to affect the transport from early endosomes to late endosomes and/or lysosomes (Bucci et al., 2000; Feng et al., 1995), also reduced the amount of *de novo* generated PrP^{Sc} at 48 hpi by 60% of the control (Fig. 7). A wild-type Rab9 is reported to be involved in the transport from late endosomes to TGN (Riederer et al., 1994). The overexpression of wild-type Rab9 reduced the amount of *de novo* generated PrP^{Sc} by 71% of the control; however, it was not clear which intracellular transport pathway was actually influenced (Fig. 7). The level of endogenous PrP^{C} in cells and cell-surface expression of PrP^{C} were not altered by overexpression of wild-type Rab9, wild-type Rab22a, a dominant-negative Rab7 mutant, or a dominant-negative Rab11a (Supplementary Fig. 1). Taken together, these results indicated that the inhibition of *de novo* generation of PrP^{Sc} was not caused by a change in the expression of PrP^{C}. These data suggested that intracellular transport along the endocytic-recycling pathway as well as the endo-lysosomal pathway is involved in the *de novo* generation of PrP^{Sc} after the inoculation of PrP-res.

Discussion

The lack of a method that can distinguish newly generated PrP^{Sc} from endogenous PrP^{C} and from inoculum-derived PrP^{Sc} was one of the obstacles to the investigation of the cellular events that mediate the *de novo* generation of PrP^{Sc} in the early stage of prion infection. Therefore, the analysis of intracellular dynamics of PrP^{Sc} just after inoculation of prions has

been limited to inoculum-derived PrP^{Sc} as descried previously (Jen et al., 2010; Magalhaes et al., 2005). Here, we solved this problem with a combination of the fluorescent-dye-labeled purified PrP-res and PrP^{Sc}-specific staining with mAb 132. This technique allowed us to distinguish newly generated PrP^{Sc} from the inoculated PrP-res within individual cells.

Considering that the inoculated PrP-res was mainly detected in late endosomes and lysosomes (Fig. 5 and 6) and that its levels decreased from 24 to 72 h after the inoculation (Fig. 4), the PrP-res directed to the endo-lysosomal pathway appeared to be degraded in late endosomes and/or lysosomes. Previous studies showed that inoculated PrP-res is transported to late endosomes/lysosomes after being taken up by SN56 cells or primary sensory neurons (Jen et al., 2010; Magalhaes et al., 2005). Purified PrP-res of the Obihiro strain, which cannot establish a persistent infection in N2a-3 cells, was also transported to late endosomes and lysosomes; the intracellular distribution of Alexa Fluor-labeled Obihiro strain-derived PrP-res was indistinguishable from that of the 22L strain-derived PrP-res and the inoculated PrP-res of Obihiro strain was degraded similarly to 22L strain-derived PrP-res (data not shown). Amyloid fibrils of the Aβ1-42 peptide were also reported to be transported via the endo-lysosomal pathway in SN56 cells (Magalhaes et al., 2005). Taken together, the trafficking of PrP-res via the endo-lysosomal pathway appears to be a general pathway for degradation of exogenously introduced macromolecules, rather than a pathway specific to PrP^{Sc} and the propagation of prions (Saftig and Klumperman, 2009).

Recently, Goold *et al*. reported that the plasma membrane is one of the sites for conversion based on results from experiments using PK-1 cells that express PrP^{C} tagged with Myc-epitope at the C-terminus (Goold et al., 2011). In this report, PrP^{Sc} was primarily generated at plasma membrane within 2 min after prion challenge. Contrary to this report, we

found no evidence that *de novo* generation of PrP^{Sc} primarily occurred at plasma membrane (Fig. 4-6). This discrepancy may be due to multiple factors, one of which includes clonal differences between these cell lines. For example, the former study showed that newly generated PrP^{Sc} attained steady levels by 2 h after inoculation of prions and approximately 20% of PK-1 cells in the cultures became PrP^{Sc}-positive. However, in the N2a-3 cells used in our study, the levels of newly generated PrP^{Sc} in the cells increased at least up to 72 hpi (Fig. 4), and most of the cells in the culture eventually became PrP^{Sc}-positive (data not shown). In addition, Goold *et al.* reported that PrP^{Sc} was detected at the cell membrane of prion-infected PK-1 cells; however, PrP^{Sc} was only weakly detected at the plasma membrane of N2a-3 cells persistently infected with prions, even when formic acid pre-treatment that was used for PrP^{Sc}-specific detection by Goold *et al.* was employed (data not shown). Finally, the differences between the two studies might be accounted for the different inocula (purified PrP-res vs. brain homogenate) used to initiate infection.

Most PrP^{C} is known to cycle between a peri-nuclear region of the cell and the plasma membrane via the endocytic-recycling pathway after being trafficked to the cell surface, but some portion of PrP^{C} is also delivered to late endosomes and lysosomes (Morris et al., 2006; Peters et al., 2003; Shyng et al., 1993). Considering that the newly generated PrP^{Sc} appeared in late endosomes, but was rarely observed in lysosomes at 24 hpi, at which time a large portion of the inoculated PrP-res was localized in late endosomes (Fig. 5 and 6), the initial conversion of PrP^{C} to PrP^{Sc} may occur in late endosomes, at least when purified PrP-res is used as inoculum. This idea is consistent with the finding that overexpression of the dominant-negative mutant of Rab7, which inhibits transport from early endosomes to late endosomes and/or lysosomes (Bucci et al., 2000; Feng et al., 1995), partly inhibited the

generation of PrP^{Sc} after the inoculation of PrP-res (reduced to 60% of the control, Fig. 7). This finding raises the possibility that although most of the inoculated PrP-res was transported to and degraded in late endosomes/lysosomes, smaller PrP-res oligomers might be generated in late endosomes during the degradation process. Such smaller PrP-res oligomers may initiate PrP^{C} conversion because smaller oligomers have greater seeding activity for the conversion of PrP^{C} and higher infectivity than do larger PrP-res aggregates (Silveira et al., 2005).

Earlier studies suggested that in cells persistently infected with prions, PrP^{Sc} is formed either on the plasma membrane or during endocytic trafficking (Borchelt et al., 1992; Caughey and Raymond, 1991; Taraboulos et al., 1992). In later years, Béranger *et al*. and Marijanovic *et al*. reported that overexpression of dominant-negative mutants of Rab4a or Rab11a, which are known to impair the transport from early endosomes to the plasma membrane (Roberts et al., 2001) or from recycling endosomes to plasma membrane (Ren et al., 1998), respectively, raised the PrP^{Sc} level (Beranger et al., 2002; Marijanovic et al., 2009). On the other hand, overexpression of wild-type Rab22a, which inhibits the transport from early endosomes to recycling endosomes (Magadan et al., 2006), reduced the PrP^{Sc} level in cells persistently infected with prions (Marijanovic et al., 2009). Based on these findings, one of the sites for PrP^{Sc} formation in cells persistently infected with prions is thought to be in the transport pathway from early endosomes to recycling endosomes. Also in the early stage of prion infection, we confirmed the inhibition of *de novo* generation of PrP^{Sc} by overexpression of a wild-type Rab22a (reduced to 69% of the control, Fig. 7), consistent with the results of Marijanovic *et al.* in persistently prion-infected cells (Marijanovic et al., 2009). However, unlike the findings in cells persistently infected with prions, the generation of PrP^{Sc} after the

inoculation of PrP-res was partly inhibited by overexpression of a dominant-negative Rab11a mutant (reduced to 62% of the control, Fig. 7). These observations suggested that the initiation of PrP^{Sc} generation shortly after PrP-res inoculation required the recycling pathway between recycling endosomes and the plasma membrane. Further studies will be required to explain the apparent inconsistency between these results. However, the newly generated PrP^{Sc} appeared at the plasma membrane and in early endosomes where the inoculated PrP-res was rarely detected at 24 hpi (Fig. 5 and 6), suggesting the involvement of the recycling pathway; therefore, either the exogenous PrP-res degraded to an undetectable level or PrP^{Sc} newly generated in late endosomes was recycled back to plasma membrane and acted as a "seed" for conversion. Moreover, the marked increase in the newly generated PrP^{Sc} at early and recycling endosomes during the following 48 h (Fig. 5 and 6) suggested that efficient generation of PrP^{Sc} occurred once the PrP^{Sc} was transferred to the endocytic-recycling pathway.

The results in this study suggest that the transfer of the inoculated PrP-res and/or newly generated PrP^{Sc} from the endo-lysosomal pathway to the endocytic-recycling pathway is important for efficient PrP^{Sc} formation after prion inoculation. One possible route of such transfer is the trafficking from the late endosomes to the plasma membrane through the TGN, a route by which cation-independent mannose-6-phosphate receptor (CIMPR) is transported (Maxfield and McGraw, 2004). CIMPR delivers hydrolase precursors from the Golgi apparatus to the endosomes and releases the hydrolases into compartments in the process of late endosome formation; CIMPR is then recycled back from late endosomes to the TGN by retrograde transport. CIMPR in the TGN is also delivered to plasma membrane (Ghosh et al., 2003). The overexpression of Rab9, which is involved in this retrograde transport of CIMPR

(Riederer et al., 1994), was reported to decrease the levels of PrP^{Sc} in cells persistently infected with prions (Gilch et al., 2009) as well as inhibit the generation of PrP^{Sc} after prion inoculation (this study), suggesting that the trafficking from late endosomes to the TGN may be involved in the generation of PrP^{Sc}.

Direct transport from late endosomes to the plasma membrane might be an alternative route. This atypical transport was reported in the trafficking of class II molecules of the major histocompatibility complex (MHC) and CD1 family molecules in antigen-presenting cells (Gelin et al., 2009; Neefjes et al., 2011). Furthermore, MHC class II molecules are also known to be transported in the process of exosomes release (Berger and Roche, 2009; Von Bartheld and Altick, 2011). Considering the facts that PrP^{C} is present on the membranes of both multivesicular bodies and intraluminal vesicles and that PrP^{Sc} as well as PrP^{C} are released from cells with exosomes (Fevrier et al., 2004), the inoculated PrP-res and/or newly generated PrP^{Sc} in late endosomes may be recycled back to the plasma membrane through multivesicular bodies via a pathway similar to MHC class II molecules. This idea is consistent with the finding that *de novo* generation of PrP^{Sc} was inhibited by the overexpression of the dominant-negative Rab11a mutant, which also inhibits the release of exosomes (Savina et al., 2002). The PrP^{Sc} recycled to the plasma membrane via these mechanisms may, in turn, contribute to *de novo* generation of PrP^{Sc} in the compartments on the endocytic-recycling pathway.

The intracellular dynamics of PrP^{Sc} in CNS neurons in the early stages after prion infection is largely unknown. It was reported that PrP^{Sc} could be detected in endosomal and lysosomal fractions prior to the detection of PrP^{Sc} in the plasma membrane fraction after intracerebral inoculation of prions (Dearmond and Bajsarowicz, 2010), and that protease-sensitive PrP^{Sc}

could be detected in early and recycling endosomes in neurons in the hippocampus during the preclinical stage of infected mice (Godsave et al., 2008). These facts suggest that there are similarities in prion propagation in neuroblastoma cells and neurons in CNS.

In this study, we showed that intracellular dynamics of inoculated PrP-res in prion-susceptible neuroblastoma cells. Our data suggest that transfer of inoculated PrP-res from endo-lysosomal pathway to endocytic-recycling pathway is involved in the initiation of efficient *de novo* generation of PrP^{Sc} in the early stage of infection (Fig. 8). However, further analyses are required for the understanding of the mechanisms of prion propagation in neurons in CNS. Experiments are underway to clarify the intracellular site for PrP^{Sc} generation in neurons in CNS using PrP^{Sc}-specific staining with mAb 132.

Materials and methods

Antibodies, expression plasmids, and reagents

Anti-PrP mouse mAbs 31C6 and 132 and a rabbit polyclonal antibody B103 were used (Horiuchi et al., 1995; Kim et al., 2004). The other commercially available primary and secondary antibodies that were used for immunoblotting and IFA are listed in Table S1. Alexa Fluor 488- and 555-conjugated Tfn and Alexa Fluor 488-conjugated LDL (Life Technologies) were used as markers for the endocytic pathway. Expression plasmids encoding EGFP-tagged Rab GTPases were prepared as described in Supplemental materials according to methods described elsewhere (Table S2, S3) (Fukuda, 2003).

Cell culture

N2a-3 cells, a subclone of the mouse neuroblastoma cell line Neuro2a, were cultured as described previously (Uryu et al., 2007).

Purification of PrP-res

PrP-res was prepared from detergent-resistant membranes as described previously (Baron et al., 2011) with slight modifications (Supplementary materials). The purification procedure included a PK treatment, so from this point forward we use the term “PrP-res” to indicate the purified, PK-treated PrP^{Sc}.

Fluorescent-dye-labeling of PrP-res

PrP-res in PBS (10 μg in 50 μl) containing 0.5% Zwittergent 3-14 was sonicated with a

Cross Ultrasonic Protein Auto Activating Instrument, ELSTEIN NP070-GOT (Nepa Gene), by 4 cycles of 15 min-sonication followed by 5 min-incubation at 4˚C prior to the fluorescent-dye labeling. The PrP-res was then mixed with 200 μg of Alexa Fluor 488 succinimidyl ester or Alexa Fluor 555 succinimidyl ester (Life Technologies) dissolved in 5 μl of anhydrous dimethyl sulfoxide (Sigma). Fluorescent-dye labeling was performed under 16 cycles of 15 min-sonication followed by 30 min-incubation at 4˚C. To quench the excess reactive dye, 3 ml of 50 mM glycine in PBS was mixed with the PrP-res and the sample was centrifuged at 45,000 rpm for 30 min at 4˚C in a S80AT3 rotor (Hitachi). The pellet was washed twice with 50 mM glycine in PBS and each wash was followed by centrifugation; the final pellet was resuspended in 100 μl of PBS with sonication. The fluorescent-dye-labeled PrP-res or unlabeled PrP-res was subjected to SDS-PAGE followed by fluorescent imaging with a Typhoon FLA 9000 (GE Healthcare) or by silver-staining with 2D-SILVER STAIN-II (Cosmo bio CO.), respectively.

Inoculation of PrP-res

Purified PrP-res or fluorescent-dye-labeled PrP-res was diluted with Opti-MEM (Life technologies), sonicated with an ELSTEIN NP070-GOT sonicator for 5 min, and centrifuged at 10,000 × *g* for 10 min to remove large PrP-res aggregates. The supernatant was diluted with Opti-MEM at 10 ng PrP-res/200 μl or 20 ng PrP-res/250 μl or 35 ng PrP-res/500 μl for use on 8-well Lab-Tek II chambered coverglass (Thermo Scientific) or 24- or 12-well plates, respectively. The culture medium for the N2a-3 cells grown on chambered coverglass or in the multi-well plates was replaced with Opti-MEM containing PrP-res, and the cells were then incubated for 2 or 6 h at 37˚C. After the incubation, the cells were washed three times

with pre-warmed PBS and then cultured in Opti-MEM containing 10% fetal bovine serum (FBS), 1% non-essential amino acids (NEAA; Gibco), and 1X penicillin-streptomycin solution (100 U/ml-100 μg/ml, PS; Gibco).

Immunofluorescence assay (IFA)

IFA, including PrP^{Sc}-specific staining, was carried out as described previously (Yamasaki et al., 2012) with some modifications. Cells were grown on 8-well Lab-Tek II chambered coverglass and all the staining procedures were carried out without a removal of the media chamber. For the double staining of cell surface PrP^{C} and PrP^{Sc}, living cells were incubated with B103 (5 μg/ml) in Opti-MEM for 15 min at 37˚C. The cells were then immediately fixed with pre-warmed 4% paraformaldehyde with 4% sucrose in PBS for 10 min and blocked with 5% FBS in PBS. Cells were then incubated with secondary antibody, and the cells were fixed again with 4% paraformaldehyde in PBS for 10 min and were subjected to the PrP^{Sc}-specific staining. To counterstain cell nuclei, cells were incubated with 5 μg/ml of 4', 6-diamidino-2-phenylindole, dilactate (DAPI; Invitrogen) in PBS at room temperature (rt) for 30 min. Finally, the media chamber was filled with PBS and confocal fluorescent images were acquired with a 63× objective lens on a Zeiss LSM700 inverted microscope and ZEN 2009 software. Z-series of the images were taken at every 0.8 μm steps from the top to bottom of the cells in the area.

Transfection

N2a-3 cells seeded onto 8-well chambered coverglass or 24-well plates at 1:5 ratio were cultured in Dulbecco's modified Eagle's medium (DMEM; ICN Biomedicals) for 2 days prior

to the inoculation of PrP-res and transfection. The cells were transfected with 2.8% Lipofectamine 2000 (Invitrogen) and 2 μg/ml expression plasmid in 250 μl (8-well chamber) or 500 μl (24-well plate) of Opti-MEM that contained 8% FBS, 0.8% NEAA, and 0.8X PS. After transfection (24 h), the medium was replaced with fresh Opti-MEM containing 10% FBS, 1% NEAA and 1X PS and cultured until used for immunoblots or IFA.

Immunoblotting and dot-blotting

Immunoblotting and dot-blotting were performed to monitor PrP and other molecules as described elsewhere (Nakamitsu et al., 2010; Uryu et al., 2007). To monitor PrP-res via immunoblotting, the protein concentration of cell lysate was adjusted to 1 mg/ml and the samples were then treated with 1 μg/ml of PK for 20 min at 37˚C. Meanwhile, to monitor Alexa Fluor 488-labeled PrP-res, PK treatment was omitted. The cell lysates were incubated with 50 μg/ml of DNase I (Roche) for 15 min at rt. Proteins were concentrated by incubating the samples with 0.3% phosphotungstic acid for 20 min at rt; this incubation was followed by centrifugation at 20,500 × g for 20 min at 4˚C.

To monitor PrP-res via dot-blotting, cell lysate equivalent to 40 μg of total protein per well was transferred onto a polyvinylidene difluoride (PVDF) membrane by dot-blotter (Bio-Rad). The PVDF membrane was treated with 20 μg/ml of PK for 1 h at 37˚C and then incubated with 1 mM Pefabloc SC for 15 min at 4˚C. The membrane was treated with 50 μg/ml of DNase I for 15 min at rt and then incubated in 3M GdnSCN for 30 min at rt. Samples on each membrane were then subjected to immuno-detection with mAb 31C6 and HRP-conjugated secondary antibody. ECL Western Blotting Detection Reagents (GE Healthcare) and a LAS-3000 chemiluminescence image analyzer (Fuji Film) were used to

visualize the immunoreactive proteins.

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Figure legends

Figure 1. Characterization of purified PrP-res.

(a) Purity of the PrP-res preparation. Purified PrP-res fractions from the brains of mice infected with the Obihiro strain (Obi-PrP-res) and the 22L strain (22L-PrP-res) were subjected to SDS-PAGE followed by silver staining and immunoblotting with mAb 31C6. (b) Purity of fluorescent-dye-labeled PrP-res. The purified 22L-PrP-res was labeled with Alexa Fluor 488 (Af488-22L-PrP-res) or Alexa Fluor 555 (Af555-22L-PrP-res). Purity and fluorescent-dye-labeling were analyzed with silver staining, immunoblotting with mAb 31C6, and fluorescence imaging. (c) Influence of sonication on purified PrP-res. Purified 22L-PrP-res was sonicated and then before and after centrifugation at 10,000 × *g* for 10 min [supernatant (Sup), precipitate (Ppt)] the PrP-res was subjected to immunoblotting with mAb 31C6. (d) PrP^{Sc}-specific staining of cells inoculated with PrP-res. N2a-3 cells were incubated with Af555-22L-PrP-res for 6 h at 37˚C, and then subjected to PrP^{Sc}-specific staining. The top panel shows the images of PrP^{Sc} detected with mAb 132 (green, left) and Af555-22L-PrP-res (red, center), and their merged image (right). The bottom panel shows the corresponding high-magnification images of the boxed regions. Cells were counterstained with DAPI (blue). Scale bar: 10 μm.

Figure 2. Intracellular localization of fluorescent-dye-labeled, purified-PrPres.

N2a-3 cells grown on chambered coverglass were transfected with expression plasmids of EGFP-tagged wild-type of Rab4a, 5a, 7, 9, 11a or 22a. Medium (200 μl) containing 10 ng of Af555-22L-PrP-res was added to cells 72 h after transfection. Time series of images were

acquired any of 15 min-duration (images were acquired every 5 seconds) within 2 h after the initiation of incubation with Af555-22L-PrP-res. Each image in a row shows a merged image of Af555-22L-PrP-res (red) and EGFP-Rab GTPase (green) with differential interference contrast (DIC) at the indicated time point. Arrows indicate examples of Af555-22L-PrP-res that became co-localized with EGFP-Rab GTPases during the period of observation. Scale bar: 5 μm.

Figure 3. Co-localization of Af555-22L-PrP-res with Tfn or LDL.

Time-lapse imaging of inoculated PrP-res and Af488-Tfn (a) or Af488-LDL (b). N2a-3 cells grown on chambered coverglass were incubated with Af555-22L-PrP-res (red) and 10 μg/ml of Af488-Tfn (green) as a marker of the endocytic-recycling pathway (a) or 4 μg/ml of Af488-LDL (green) as a marker of the endo-lysosomal pathway (b). Time-lapse images were acquired any of 15 min-duration (images were acquired every 5 seconds) within 2 h after the initiation of incubation with PrP-res. Each image within a row shows a merged image of Af555-22L-PrP-res (red) and Af488-Tfn or Af488-LDL (green) with the DIC at the indicated time point. Arrows indicate examples of Af555-22L-PrP-res that became co-localized with Af488-Tfn (a) or Af488-LDL (b) in the period of observation. (c) Co-localization of Af555-22L-PrP-res with Af488-Tfn or Af488-LDL. N2a-3 cells were incubated with Af555-22L-PrP-res and Af488-Tfn or Af488-LDL for 6 h at 37˚C. After the removal of excess Af555-22L-PrP-res, the cells were subjected to live-cell imaging (0 h). To monitor co-localization of PrP-res one day after inoculation, N2a-3 cells were incubated with Af555-22L-PrP-res for 6 h. After the removal of excess Af555-22L-PrP-res, the cells were further incubated for 24 h at 37˚C. The cells were then incubated for an additional 6 h in the

presence of Af488-Tfn or Af488-LDL before imaging (30 h). The right-most column shows the merged images of Af488-Tfn (green) or Af488-LDL (green), Af555-22L-PrP-res (red), and DIC. Arrows indicate examples of Af555-22L-PrP-res co-localized with Af488-Tfn or Af488-LDL. Scale bar: 5 μm. The graph on the right shows ratios of Af555-22L-PrP-res signals co-localized with Af488-Tfn or Af488-LDL signals relative to sum of the Af555-22L-PrP-res signals (methods for the calculation of the co-localization ratio are described in the Supplementary information). Mean and SD of the values acquired from 15 fields of view are depicted. Total numbers of foci and cells used for co-localization statistics from the 15 view fields were listed in Table S4.

Figure 4. Kinetics of inoculated PrP-res and *de novo* generation of PrP^{Sc}.

(a) The levels of inoculated PrP-res and total PrP-res. N2a-3 cells grown on 12-well plates were inoculated with Af488-22L-PrP-res. After the inoculation, the cells were cultured for the indicated period and then subjected to immunoblotting. As a control for detection of PrP-res, mock-infected cells were prepared (M). PK-untreated samples equivalent to 100 μg of total protein per lane were loaded to detect Af488-22L-PrP-res with anti-Alexa Fluor 488 antibody (Alexa), while PK-digested samples equivalent to 100 μg of total protein were also loaded to monitor total PrP-res, which contained inoculated PrP-res and newly generated PrP-res with anti-PrP antibody mAb 31C6 (PrP-res). β-actin was used as an internal control. Bands corresponding to monomeric and dimeric PrP^{Sc} (indicated by the square bracket) were quantified. The arrowhead indicates the mono-glycosylated form of PrP-res. The graph on the right shows the result of a quantitative analysis. Black bars indicate the inoculated PrP-res levels relative to that at 0 h and gray line indicates the total PrP-res levels relative to that at 0

h. Mean and SD of 3 independent experiments are shown. (b) Live-cell image of Af555-22L-PrP-res. N2a-3 cells grown on chambered coverglass were inoculated with Af555-22L-PrP-res and then cultured for the indicated time. Merged images containing Af555-22L-PrP-res (red) and DIC are shown. (c) Discrimination of newly generated PrP^{Sc} from inoculated PrP-res in a single cell. The top panel shows the merged images of signals of Af555-22L-PrP-res (red), PrP^{Sc} (green) and DAPI-stained nuclei (blue). The bottom panel shows the corresponding high-magnification images of the boxed region. Arrowheads indicate examples of inoculated PrP-res that was detected via both Alexa Fluor 555 (red, directly coupled to purified 22L-PrP-res) and mAb 132 (green, indirect immuno-staining with Alexa Fluor 488-conjugated secondary antibody). As a result, inoculated PrP-res can be detected as yellow. Arrows indicate examples of newly generated PrP^{Sc} that was detected only with mAb 132 (green). Scale bar: 5 μm.

Figure 5. Kinetics of the intracellular localization of inoculated PrP-res and newly generated PrP^{Sc}.

N2a-3 cells inoculated with Af555-22L-PrP-res were cultured for the indicated time and subjected to double-staining of PrP^{Sc} with mAb 132 and an organelle marker molecule as indicated. For staining of PrP^{C} at cell surface, cells were incubated with B103 antiserum prior to the fixation for PrP^{Sc}-specific detection. Alexa Fluor 647-conjugated secondary antibody was used to stain the marker molecules. The cell nuclei were counterstained with DAPI. The upper left image in each panel consists of four images shows a lower magnification view of a merged image of organelle marker molecule (red), PrP^{Sc} (green), Af555-22L-PrP-res (cyan), and nuclei (blue). The other three images are the corresponding high-magnification image of

the boxed region for the merged image of organelle marker and nuclei (bottom left), for the merged image of PrP^{Sc}, Af555-22L-PrP-res and nuclei (upper right), and for the merged images of the organelle marker, PrP^{Sc}, Af555-22L-PrP-res and nuclei (bottom right). Arrowheads indicate representative co-localization of Af555-22L-PrP-res with organelle marker molecules, while arrows indicate that of newly generated PrP^{Sc} with organelle marker molecules. Arrowheads with asterisks indicates AF555-22L-PrP-res that was not co-localized with organelle marker molecules, while arrows with asterisks indicate newly generated PrP^{Sc} that was not co-localized with organelle marker molecules. Co-localization areas were defined as pixels that were positive for both PrP^{Sc} and organelle marker signals, or that were positive for both Af555-22L-PrP-res and organelle marker signals. Scale bars: 5 μm.

Figure 6. Co-localization statistics.

Co-localization analysis of the images shown in Fig. 5 was carried out as described in the Supplementary materials. (a) Ratio of the Af555-22L-PrP-res signals co-localized with each marker to the sum of the Af555-22L-PrP-res signals. (b) Ratio of the newly generated PrP^{Sc} signals co-localized with each marker to the sum of newly generated PrP^{Sc} signals. Mean and SD of the value acquired in 5 fields of view are shown. Total numbers of foci and cells used for co-localization statistics from the 5 view fields were listed in Table S5.

Figure 7. Effect of overexpression of Rab GTPases on the *de novo* generation of PrP^{Sc}.

N2a-3 cells grown in a 24-well plate were inoculated with unlabeled 22L-PrP-res and were incubated for 6 h. After washing the cells, an expression plasmid encoding an EGFP-tagged wild-type (Wt) Rab GTPase, Rab4a, Rab5a, Rab7, Rab9, Rab11a or Rab22a, or an

EGFP-tagged dominant-negative mutant (DN) of a Rab GTPases, Rab4aS22N (Roberts et al., 2001), Rab5aS34N (Stenmark et al., 1994), Rab7T22N (Feng et al., 1995), Rab9S21N (Riederer et al., 1994), Rab11aS25N (Ren et al., 1998) or Rab22aS19N (Weigert et al., 2004), was introduced. The expression vector pEGFP-C1 (Clontech) was used as a control. At 48 h after transfection, the cells were processed for dot-blotting to monitor PrP-res. A representative image of a dot-blot is shown on the top, and the graph below shows the levels of PrP-res relative to that of control plasmid-transfected cells (EGFP). Mean and SD of 4 independent experiments are depicted. Asterisks indicate a significant decrease compared to the control (Student's *t*-test, $p<0.05$).

Figure 8. Summary of intracellular dynamics of inoculated PrP^{Sc} and *de novo* generation of PrP^{Sc} in early stage of prion infection.

Exogenously introduced PrP^{Sc} (red parallelograms) is internalized into a cell (i). Although inoculated PrP^{Sc} is transported throughout endocytic compartment immediately after internalization, most of the inoculated PrP^{Sc} eventually directed to endo-lysosomal pathway (indicated as red arrows) and is delivered to lysosomes and degraded (iii). Conversion of PrP^{C} (pink ellipse) to PrP^{Sc} (pink parallelogram) may be initiated by fragmented inoculated PrP^{Sc} (a smaller PrP^{Sc} oligomer) that is generated during the transport on the endo-lysosomal pathway (ii). A smaller PrP^{Sc} oligomer generated by fragmentation of inoculated PrP^{Sc} and/or newly generated PrP^{Sc} is transferred from the endo-lysosomal pathway to the endocytic-recycling pathway (iv). Once PrP^{Sc} that is capable of inducing conversion (blue parallelograms) is transferred to endocytic-recycling pathway (indicated as blue arrows) initiates efficient PrP^{Sc} formation that leads to the establishment of prion infection. The sites where efficient

conversion occurs are thought to be early endosomes and recycling endosomes (v).