Stimulation of PrP\textsuperscript{C} retrograde transport towards the Endoplasmic Reticulum increases accumulation of PrP\textsuperscript{Sc} in prion-infected cells

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Running title

Retrograde transport and PrP$^\text{Sc}$ production
Abstract

Prion diseases are fatal and transmissible neurodegenerative disorders characterized by the accumulation of an abnormally folded isoform of the cellular prion protein (PrP\textsuperscript{C}) denoted PrP\textsuperscript{Sc}. In order to identify intracellular organelles involved in PrP\textsuperscript{Sc} formation, we studied the role of the Ras-related GTP-binding proteins Rab4 and Rab6a in intracellular trafficking of the prion protein and production of PrP\textsuperscript{Sc}. When a dominant-negative Rab4 mutant or a constitutively active GTP-bound Rab6a protein was over-expressed in prion-infected neuroblastoma N2a cells, there was a marked increase of PrP\textsuperscript{Sc} formation. By immunofluorescence and cell fractionation studies we have shown that expression of Rab6a-GTP delocalizes PrP within intracellular compartments leading to an accumulation in the endoplasmic reticulum (ER). These results suggest that prion protein can be subjected to retrograde transport towards the ER and that this compartment may play a significant role in PrP\textsuperscript{Sc} conversion.
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 exemplify a novel mechanism of biological information transfer characterized by the generation of an abnormally folded isoform of the cellular prion protein (PrPC), denoted PrPSc, which represents the major component of infectious prion particles (1). PrPC is a neuronal membrane glycoprotein whose function has not been fully characterized; PrPC is rich in $\alpha$-helical regions but can be converted into PrPSc, a protease-resistant isoform rich in $\beta$-sheet content that accumulates in the brain of infected organisms (2). It has been postulated that PrPSc may physically interact with PrPC, acting as a ‘nucleus’ or template for the formation of new PrPSc molecules. During biogenesis, PrPC transits through the secretory pathway and is modified both by glycosylation and addition of a C-terminal GPI anchor. It is delivered to the cell surface clustered in detergent insoluble domains (DIM) as has been observed for other GPI anchored proteins and signalling molecules (3,4). The PrP protein is then rapidly endocytosed and recycled back to the cell surface. During this process, PrPC is cleaved, probably in an acidic cellular compartment. In cellular models, the conversion of PrPC into PrPSc is thought to occur after PrPC has reached the plasma membrane and has been re-internalized for degradation (5, 6). The molecular mechanisms underlying the conversion reaction are enigmatic and the precise intracellular compartment where it occurs remains unknown.

Cells maintain several dynamic properties to internalize and secrete proteins in different ways. The traffic of membranes between organelles occurs through vesicular or tubular intermediates that selectively convey proteins and lipids from one compartment to another under the control of specific proteins. In this context, small GTPases of the Rab family are believed to play a role of insuring accurate targeting or docking of transport vesicles with their acceptor membranes (7-9). They operate as molecular switches by interacting with different sets of proteins in the inactive GDP or active GTP bound states (review in (10)). Individual members of the Rab family are
uniquely localized in specific membrane compartments and control the direction and/or specificity of particular steps in intracellular protein trafficking (Figure 1). The roles of specific Rab proteins have been partially defined by studies involving over-expression of mutant Rab proteins with defective guanine nucleotide binding properties.

The exact subcellular sites of the processing events involved in the genesis of PrPSc remain to be determined. It is likely that many, if not all, of the steps in the intracellular trafficking of PrP are mediated by distinct members of the Rab family. Consequently, functional perturbation of Rab proteins known to be localized in specific subcellular compartments may help to define the routes by which PrPC is converted into PrPSc. As a first step in testing this approach, we have examined the effects of dominant-negative or constitutively active mutations of Rab4 and Rab6a proteins on the formation of PrPSc in a prion-infected neuroblastoma cell line.

Rab4 protein has been implicated in the regulation of membrane recycling from early endosomes to the recycling compartment or directly to the plasma membrane (11, 12) and may be involved in the plasma membrane recycling of PrPC. Rab6a stimulates retrograde transport at the level of the Golgi and induces a progressive, microtubule-dependent redistribution of Golgi resident proteins to the endoplasmic reticulum (ER) (13,14). When the dominant-negative Rab4-GDP or the constitutively activated Rab6a-GTP mutants were over-expressed in prion infected N2a cells, we detected a marked increase in the conversion of PrPC to the pathogenic form PrPSc. This effect was observed both with endogenous PrPC and with an exogenous 3F4-tagged-MoPrP (15). Immunofluorescence and cell fractionation studies demonstrated an accumulation of PrP in the endoplasmic reticulum in Rab6a-GTP expressing cells. Our data suggest that retro-transport of PrPC towards the ER increases the production of PrPSc suggesting that this organelle plays an important role in PrPSc formation.
Material and Methods

Reagents and antibodies

Cell culture reagents (Opti-MEM, L-Glutamine and Trypsin) were from Life Technologies Inc. and foetal calf serum from Bio-Whitaker. Secondary antibodies were from Jackson Immunoresearch (West Grove, PA, USA). All other reagents were from Sigma. Pri308, SAF 32, 60, 69 and 70 are five mAb produced by the group of J.Grassi (CEA Saclay, France). A mixture consisting of an equal volume of ascitic fluids of SAF 60, 69 and 70 antibodies was used to improve PrP detection, namely SAF mix. Anti-PrP 45-66 polyclonal antibodies were a kind gift from Dr David Harris. Anti-Myc, anti-PDI and anti-GS28 monoclonal antibodies were purchased from TEBU (Le Perray-en-Yvelines, France).

Cell culture

N2a neuroblastoma cells stably transfected with wild-type MoPrP and infected with Chandler (N2aMoPrP-Ch) or 22L (N2aMoPrP-22L) prion strains have been described previously (16). These cells were routinely culture in Opti-MEM (Life Technologies Inc.) supplemented with 10% heat-inactivated foetal calf serum and penicillin-streptomycin and maintained at 37°C in 5% CO2 in a biohazard Level 3 laboratory.

Transfection assays, proteinase K digestion and Western blotting

Rab4wt, Rab4S22N and Rab4Q67L cDNAs were a kind gift from Dr. Mary McCaffrey. Rab4 and Rab6awt, Rab6aT27N and Rab6aQ72L cDNAs were subcloned in the eukaryotic vector pRK5myc downstream and in-frame with the Myc epitope MEQKLISEEDL and sequenced verified. N2aMoPrP-22L cells were transfected using Fugene 6 (Roche Molecular Biochemicals) or Lipofectamine (Life Technologies Inc.) according to the manufacturer’s instructions. Four days after transfection, cells were washed in PBS and lysed for 20 minutes at 4°C in Triton/DOC.
lysis buffer (150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 50 mM Tris pH 7.5 and protease inhibitors). After 3 min of centrifugation at 6,000 rpm, the supernatant was collected and assayed for total protein content with a BCA Protein Assay kit (Pierce). Protein concentration was adjusted with lysis buffer. For detection of PrP$_{Sc}$, equivalent volumes of samples were digested with 16µg of proteinase K per mg of protein at 37°C for 30 min, and the digestion was stopped by incubation with Pefablock (1 mM) for 5 min on ice. The samples were centrifuged at 14,000 rpm for 45 min at 4°C and the pellets re-suspended in 25 µl of SDS loading buffer and boiled for 5 min. Proteins were separated by SDS-PAGE in 12% acrylamide gels and transferred onto Immobilon-P membrane (Millipore) in Towbin buffer containing 10% ethanol. The membrane was blocked with 5% non-fat dry milk in TBST (0.1% Tween 20, 100 mM NaCl, 10 mM Tris-HCl; pH 7.8) for 1 hr at room temperature. After an overnight incubation at 4°C with primary antibodies (anti-PrP SAF mix or SAF32 diluted 1/300 in 5% milk in TTBS, anti-PDI or anti-GS28 diluted 1/500 in TTBS) the membrane was washed 4 times in TTBS and incubated for 1 hr with an anti-mouse horseradish peroxydase conjugate (Amersham Pharmacia Biotech) diluted 1/5000 in 5% milk in TTBS. The membrane was washed 4 times with TTBS and developed using enhanced chemiluminescence (ECL). Gel bands were quantified using Sigma Scan Image software.

**Cell fractionation**

To separate and enrich Golgi and ER membranes, N2aMoPrP-22L cells were homogenised by using a stainless steel ball-bearing homogeniser (HGM precision engineering, Heidelberg, Germany) in 0.25 M sucrose, 10 mM Tris-HCl pH 7.4, 1 mM magnesium acetate, and a protease inhibitor mixture in a final concentration of 1 volume of cell pellet per 5 volumes of homogenising medium. Sucrose gradients were performed as described in (17). 1 ml fractions were collected from the top of each gradient, assayed for protein content and methanol-precipitated. After centrifugation at 14,000 rpm for 20 min, the pellets were re-suspended in SDS loading buffer. PrP was assayed by running fractions on 12% SDS PAGE, transferring the proteins onto Immobilon membranes and performing Western blot analysis with Saf32 antibodies.
**Immunofluorescence**

N2aMoPrP-22L cells were fixed in 3.7% formaldehyde in PBS for 10 min, washed 3 times with PBS and permeabilised in 0.1% Triton X-100-PBS for 3 min. Permeabilised cells were incubated for 1 hr at room temperature in 0.2% BSA in PBS (PBS-BSA), and for 1 hr at 37°C with primary antibodies diluted in PBS-BSA: anti-PrP 45-66 polyclonal antibodies (1/300), anti-PDI (1/500) or anti-Myc 9E10 mAb (1/200). After three washes in PBS-BSA, cells were further incubated with fluorescein conjugated anti-rabbit antibodies (dilution 1/50) and Texas Red conjugated anti-mouse antibodies (dilution 1/50) for 1 hr at 37°C. DNA was stained with Hoeschst 33286. Cells were washed and mounted in FluorSave reagent (Calbiochem). Images were collected and processed on a Zeiss Axiophot.

**Results**

*Effect of Rab proteins on endogenous PrPSc formation*

In order to interfere with PrP intracellular trafficking and processing, prion infected neuroblastoma N2a cells over expressing mouse PrP (N2aMoPrP-Ch and N2aMoPrP-22L) were transfected with expression vectors encoding Myc-Rab4WT, Myc-Rab6aWT, the dominant negative mutants Myc-Rab4S22N and Myc-Rab6aT27N and the GTPase-deficient mutants Myc-Rab4Q67L and Myc-Rab6aQ72L proteins. Expression levels of the Myc-Rab4 and Myc-Rab6a wild-type and mutant proteins ranged from 3- to 5- fold over the endogenous Rab proteins (data not shown). Four days after transfection, cells were lysed and equal amounts of proteins were digested with proteinase K. The relative amounts of PrPSc detected by immunoblot analysis were measured in the different samples. Rab protein expression did not have any major effect on PrP production (data not shown), but for accurate data analysis the values were normalized to the total intracellular PrP in each sample. Compared with cultures that were transfected with the expression vector alone, cells over expressing Rab4S22N and Rab6aQ72L
showed a dramatic increase of PrP\textsuperscript{Sc} production (Fig.2A and B). No significant effect was observed in cells expressing Rab4Q67L or Rab6aT27N. Similar results were obtained with cells infected by the two different prion strains (Chandler and 22L). These observations provide the first indication that over expression of mutant Rab proteins might affect PrP intracellular trafficking and therefore the conversion of PrP\textsuperscript{C} into PrP\textsuperscript{Sc}.

To validate these results, we determined whether expression of mutant Rab proteins could interfere with the pathogenic conversion of a \textit{de novo} synthesized PrP protein. N2aMoPrP-Ch cells infected by the Chandler prion strain were co-transfected with Myc-Rab4S22N, Myc-Rab6a\textsuperscript{WT} or Myc-Rab6aQ72L expression vectors and a 3F4-tagged MoPrP plasmid. 4 days after transfection, total cellular lysates were subjected to proteinase K digestion and exogenous newly converted PrP\textsuperscript{Sc} was revealed by immunoblot with monoclonal antibodies directed against the 3F4 epitope (Pri308). Expression levels of 3F4-MoPrP were similar in each condition (not shown). Data presented on figure 2 (C and D) demonstrated an increased accumulation of exogenous 3F4-PrP\textsuperscript{Sc} in cells expressing Myc-Rab4S22N, Myc-Rab6a\textsuperscript{WT} and Myc-Rab6aQ72L.

Combined with the data observed with endogenous PrP, these results strongly suggest that impairing the plasma membrane recycling of PrP (by over expressing Rab4S22N) and stimulating the retrograde transport of proteins towards the ER (by expressing Rab6a\textsuperscript{WT} and Rab6aQ72L) increased the formation of the PrP\textsuperscript{Sc} conformation. These results are in agreement with previous findings showing that PrP\textsuperscript{Sc} conversion occurs within intracellular compartments and suggest that PrP\textsuperscript{Sc} formation may involve a retrograde transport of PrP molecules into the ER.

\textit{Rab6aQ72L expression induces accumulation of PrP in intracellular compartments.}

Before proceeding with further analysis of PrP\textsuperscript{Sc} formation in cells expressing mutant Rab
proteins, an immunofluorescence study was conducted to determine the sub-cellular localisation of PrP in the presence of Rab proteins. Because the expression of Rab6a\textsuperscript{Q72L} had the most dramatic effect on PrP\textsuperscript{C} conversion into PrP\textsuperscript{Sc}, we decided to focus our study on the role of this Rab6a mutant protein on PrP trafficking. N2a MoPrP-22L cells, that had been transfected with Myc-Rab6a\textsuperscript{Q72L}, were analyzed by indirect immunofluorescence. The overall transfection efficiency for the Myc-Rab6a\textsuperscript{Q72L} vector was estimated to be approximately 90%. As shown in Figure 3, PrP is excluded from the cell surface in Myc-Rab6a\textsuperscript{Q72L} expressing cells and accumulates in an intracellular compartment partially co-localized with Rab6a in the Golgi apparatus (A-F). We used PDI (protein disulphide isomerase) as an ER marker in double labeling immunofluorescence experiments. PDI, a protein resident in the lumen of the endoplasmic reticulum, is distributed in a reticular pattern throughout the cytoplasm. We found that intracellular PrP showed a widespread cytoplasmic staining pattern that co-localized with PDI, producing a yellow colour throughout the cytoplasm in a merge image (Figure 3 G-I). These results demonstrate that expression of the Rab6a GTP-bound form in N2a cells induces an altered distribution of PrP towards intracellular compartments, and that intracellular PrP outside of the Golgi co-localizes with an ER marker. These results were independent of the N2a cell line because similar results were observed with CHO cells over-expressing MoPrP (data not shown). Interestingly, we can also detect the presence of PrP in the nuclei of the cells, presumably in the nucleoli, as already described (18, 19)

The intracellular localization of PrP in Rab6a\textsuperscript{Q72L} expressing cells was also determined by sub-cellular fractionation on sucrose density gradients. GS28 and PDI were used as markers for Golgi and ER fractions respectively. Mock-transfected or over-expressing Myc-Rab6a\textsuperscript{Q72L} N2aMoPrP-22L cells were fractionated on an equilibrium sucrose gradient and the distribution of total PrP was determined by immunoblotting the subcellular fractions with anti-PrP antibodies. Figure 4 shows that PrP was most abundant in the ER-enriched fractions in Rab6a\textsuperscript{Q72L} transfected cells.
Our results demonstrate that stimulation of retrograde transport by Rab6aQ72L in N2a cells induced an intracellular redistribution of the prion protein within the ER compartment, showing for the first time that PrP proteins can be subject to an ER retrograde transport. Furthermore, we have determined that accumulation of PrP molecules in the ER is accompanied by an increase of PrPSc formation.
**PrPSc accumulated with Rab6aQ72L does not correspond to ERAD-proteasome dependant PrP molecules**

Recent studies have shown that wild-type PrPC molecules were degraded through the ER-associated degradation (ERAD)-proteasome pathway (20,21). The ERAD-proteasome pathway is involved in the degradation of incorrectly folded proteins which failed to pass the ER ‘quality control’. Cells treated with proteasome inhibitors accumulate partially protease resistant insoluble PrP molecules. The PK resistant PrP molecules detected in the presence of Rab6aQ72L in infected N2aMoPrP-22L cells could therefore correspond to an accumulation in the ER of PrPC molecules which failed to be degraded by the ERAD pathway. To address this question, non infected N2aMoPrP cells were transfected with Myc-Rab6aQ72L or incubated for 12 hours with 150µM of the proteasome inhibitor ALLN (N-acetyl-leucinal-leucinal-norleucinal). Lysates were subjected to partial proteolysis (5, 10 or 16 µg/mg proteins proteinase K, 30 min at 37°C) before western-blotting with Saf Mix antibodies (Figure 5A). In mock or Rab6aQ72L-transfected cells PrPC was completely digested with 10 or 16 µg/mg of proteinase K, suggesting that abnormally folded PrP proteins are efficiently degraded by the proteasome system. When the ERAD-proteasome pathway is inhibited, partially protease resistant PrP species can be detected (ALLN-treated cells). Equal amounts of PrPC were detected in the different samples before PK digestion (Figure 5B). Figure 5C show the expression of Myc-Rab6aQ72L in the transfected cells. We conclude that the expression of Rab6aQ72L in prion-infected N2aMoPrP cells induced accumulation of PrpSc and not PK resistant PrP molecules which failed to pass the ERAD proteasome system.

**Discussion**

A major conformational change is thought to be a key event in the conversion of PrPC into the abnormal PrpSc isoform. To understand the molecular events involved in the pathogenesis of
prion diseases, it is important to know which subcellular compartments are implicated in the conversion mechanisms. PrPC is synthesized in the rough endoplasmic reticulum and transits through the Golgi apparatus on its way to the cell surface. During its biosynthesis, PrPC is subject to several post-translational modifications: N-glycosylation at two sites, formation of a disulfide bond and attachment of a GPI anchor. PrPC is addressed to the cell surface where it is attached by its GPI anchor. PrPC does not remain on the cell surface but constitutively cycles between the plasma membrane and an endocytic compartment from which most of the PrPC is recycled back to the cell surface. This endocytic recycling pathway is of interest because it may be the route along which conversion of PrPC to PrPSc takes place. It has been hypothesized that detergent-resistant micro-domains of the plasma membrane ("rafts") may be involved in formation of PrPSc (3,4,22,23). However, kinetic studies of mutant PrP molecules synthesized in CHO cells suggest that individual steps in formation of PrPSc may take place in at least two different cellular locations (24). In fact mutant proteins are already abnormal following synthesis in the ER while detergent insolubility and protease resistance properties are acquired after arrival of the proteins at the cell surface. Because the transport routes which determine PrPC endocytosis and PrPSc conversion remain elusive, we studied the effect of Rab mutant proteins on PrPSc formation. Rab proteins are distributed in distinct intracellular compartments and regulate specific tethering/docking of incoming vesicles to the correct target organelle (10). As a first approach, we used Rab4, Rab6 and Rab9 wild-type and mutant proteins to interfere with PrP intracellular trafficking in prion-infected N2a cells.

Rab4 proteins are localized in early and recycling endosomes and are implicated in early endosomes protein sorting and recycling to the cell surface. We have found that expression of the GDP-bound Rab4S22N mutant in prion-infected N2a cells impairs plasma membrane recycling and resulted in an accumulation of PrPSc. These results suggest that PrPSc formation does not require cell surface recycling and occurs within an intracellular compartment, in agreement with several publications demonstrating that an endocytic pathway was involved in the generation of
PrPSc in scrapie-infected N2a cells (25, 26).

In order to interfere with downstream steps of PrP trafficking, we studied the effect of wild-type or mutant Rab6a proteins on PrP conversion. Extensive studies have documented the localisation of Rab6a in the \textit{trans}-Golgi cisternae and \textit{trans}-Golgi network in mammalian cells. Highly over-expressed active forms of Rab6a (Q72L mutant or wild-type) stimulate retrograde transport from post-Golgi vesicles back to the \textit{trans}-Golgi network, progressively relocating Golgi residents to the ER (13, 14). Surprisingly, we found that over-expression of a wild-type or a constitutively active form of the Rab6a protein in prion-infected N2a cells resulted in a marked enhancement of PrPSc production. Using immunofluorescence and subcellular fractionation techniques, we demonstrated that over-expression of Rab6a-GTP alters the sub-cellular localization of PrP, shifting the protein from the cell surface to intracellular compartments including the ER. Previous investigations on intracellular trafficking of mammalian PrPC in living cells have shown that the protein, once internalized, is targeted to membranous structures close to the nucleus (27) and reminiscent of the Golgi apparatus (28). Based on the model of Rab6a function in intra-Golgi trafficking, we propose that Rab6a-GTP stimulates retrograde transport of PrP molecules within the \textit{trans}-Golgi compartment towards the ER. A possible physiological retro-transport of PrP\textsuperscript{C} into the ER has been already suggested. Thus, post-Golgi PrP species are mis-located into the cytosol in cells following treatment with proteasome inhibitors (20). Likewise, the truncated protein PrP\textsuperscript{Q160Stop} is prevented from leaving the Golgi apparatus and transported instead to the cytosol and nucleus (29). This retro-transport process is believed to occur only in the ER, suggesting that PrP\textsuperscript{C}, like other proteins (30), can recycle into the ER presumably by a Rab6a controlled pathway. It will be important to understand the intracellular retrograde pathway of PrP\textsuperscript{C} during endocytosis. Expression of Rab9-GDP and GTP bound mutant proteins did not influence PrPSc formation (F.B., unpublished observation), arguing against a transport of PrP\textsuperscript{C} from the late endosomes to the Golgi apparatus (31, 32). Whether PrP\textsuperscript{C} is transported to the Golgi directly from the early endosomes or the plasma membrane or downstream in the endocytic pathway from a lysosome remains to be determined.
It is interesting to speculate that PrP may be transported from the cell surface to the endoplasmic reticulum through a Rab6a regulated transport pathway. Our data are consistent with this possibility, but do not directly demonstrate it. This pathway has been previously postulated to be used for degradation of trans-membrane proteins that have been targeted for proteasome-mediated degradation in the cytosol (33). Most proteins are subject to a stringent surveillance mechanism that causes retention of misfolded forms in the ER. Proteins retained in the ER are then eliminated by reverse translocation into the cytoplasm, followed by proteasome degradation. Recent data demonstrate that several misfolded mutants of PrP^C are partially retained in the endoplasmic reticulum (34) and, as well as wild-type PrP^C, are degraded through the ERAD-proteasome pathway (20, 21). Another hypothesis of Rab6a-induced PrP^Sc formation could concern the accumulation of misfolded PrP^C proteins in the ER due to Rab6a-GTP expression. In non-infected N2a cells, proteasome inhibitors induce the appearance of detergent-insoluble and proteinase K resistant PrP^C. Such a PrP^C form could not be detected in non-infected N2a cells over-expressing Rab6a-GTP, eliminating this possibility. Therefore, the increase of detected PrP^Sc in prion-infected cells expressing Rab6a wild-type or GTP bound proteins reflects a real PrP^Sc conversion and not an accumulation of misfolded PrP^C.

The increase of PrP^Sc production described after stimulation of Rab6a-controled retrograde transport suggest either that a certain proportion of PrP^Sc remains in the ER and can induce a conformational change of retro-transported PrP^C, or that Rab6a-GTP stimulates PrP^Sc retrograde transport towards the ER where it can seed the conversion of nascent PrP^C molecules. The initial conformational change leading to PrP^Sc formation would then occur just after synthesis as already suggested following the observation that mutated PrP molecules become abnormal in the ER after their synthesis (24).

Gilch et al have shown recently that intracellular re-routing of prion protein prevents propagation of PrP^Sc (35). In this study, the compound Suramin completely prevented the plasma membrane
localization of PrP, which was re-routed directly to acidic compartments. In the light of these data and our results, it seems that plasma membrane localization of the prion protein, followed by endocytosis and retrograde transport towards the ER are necessary to induce its conversion. If any of these pathways is blocked, either with Suramin (35), Brefeldin A (26) or a dominant negative mutant of dynamin abolishing endocytosis (F.B., unpublished observation), PrP$_{Sc}$ formation is diminished.

In several studies determining the subcellular localization of mutant PrP molecules, ER retention seems to be a common feature in experimental familial prion diseases (28, 36-38). Whether ER located PrP molecules are involved in cellular neurodegeneration remains to be established. Retention of abnormal proteins in the ER is known to trigger stress response pathways (39) and could explain the pathogenic effects of mutant PrPs. Furthermore, a newly described CtmPrP, a trans-membrane form of PrP that is thought to be involved in prion-induced neurodegeneration, is completely retained in the ER (40). Multiple lines of evidence demonstrate that many pathogenic mutations interfere with normal PrP trafficking, and that an abnormal retention of PrP protein in the ER may contribute to prion-induced neurodegeneration. Our results shed some light on PrP$_{Sc}$ conversion mechanism by showing that PrP molecules can be subjected to retrograde transport towards the ER, and that stimulation of this translocation leads to an accumulation of PrP$_{Sc}$, demonstrating for the first time that the endoplasmic reticulum may play a role in PrP$_{Sc}$ formation.

**Figure Legends**

**Figure 1**: Role of different Rab family members on intracellular trafficking. Rab5 controls internalization of membrane proteins via clathrin-coated endocytic vesicles, Rab4 mediates sorting and recycling in early endosomes, Rab6a regulates retrograde *trans*-Golgi transport, Rab7 plays a role in protein degradation while Rab9 controls late endosomes to Golgi transport.
**Figure 2.** Rab4-GDP and Rab6a-GTP expression stimulates PrP\(^\text{Sc}\) production. Prion-infected N2a MoPrP (22L and Ch) cells were transfected with an empty pRK5 plasmid (control) or Rab proteins expression vectors. Four days after transfection, cells were lysed and equal amounts of total proteins content were treated with proteinase K for 30 min at 37°C. A: PrP\(^\text{Sc}\) was detected by Western blot with Saf Mix antibodies. B: Quantitative analysis of the autoradiography using Sigma Scan Image software. Ratios of PrP\(^\text{Sc}\) in Rab transfected cells were normalized to control (100%). The average of three independent experiments is shown; error bars indicate standard deviation.

N2a MoPrP-22L cells were co-transfected with a 3F4MoPrP plasmid and pRK5 (control) or Rab4S22N, Rab6a\(^{\text{WT}}\) and Rab6a\(^{\text{Q72L}}\) expression vectors. C: 3F4-MoPrP\(^\text{Sc}\) was detected by Western blot with Pri308 monoclonal antibodies. D: Quantitative analysis of the autoradiography. Ratios of 3F4-MoPrP\(^\text{Sc}\) in Rab transfected cells were normalized to control (100%).

**Figure 3.** PrP has an intracellular distribution and partially co-localizes with the ER in Rab6a-GTP expressing cells. N2aMoPrP-22L cells expressing Rab6a\(^{\text{Q72L}}\) were fixed, permeabilised and stained with rabbit anti PrP 45-66 antibodies (A, D, G) and mouse anti Myc antibody (B, E) or mouse anti-PDI antibody (H), followed by FITC-conjugated anti-rabbit and Texas Red-conjugated anti-mouse secondary antibodies. Cells were viewed with green excitation/emission settings to detect PrP and with red excitation/emission settings to detect Myc-Rab6a-GTP or PDI. Merged green and red images are shown in C, F and I. White arrow (D-F) indicates the staining of a non-expressing Rab6-GTP cell.

**Figure 4.** Localization of PrP by sucrose gradient fractionation. N2aMoPrP-22L cells were transfected with pRK5 plasmid (Mock) or Myc-Rab6a\(^{\text{Q72L}}\) expressing vector. Cell homogenates were fractionated on a sucrose gradient (see Materials and Methods). A: Proteins from each fraction analysed by Western blot using Saf32 monoclonal antibodies. For each fraction, the sucrose and protein concentrations were determined, respectively by refractometry and BCA protein assay. PDI was used as a marker for the endoplasmic reticulum fractions.
Fraction 1 corresponds to the top of the gradient. B: Quantitative analysis of the autoradiography. The graph represents the percentage of PrP in each fraction, either from mock transfected cells (dotted white bars) or Rab6aQ72L expressing cells (dotted black bars).

**Figure 5** Rab6aQ72L-induced PrPSc formation do not correspond to ERAD-proteasome dependant PrP molecules. Non-infected N2aMoPrP cells were transfected with pRK5 (control) or Rab6aQ72L expression vectors or treated for 12 h with 150µM ALLN. A: Equal protein amounts of each cell lysates were incubated with different concentrations of proteinase K (5, 10 or 16µg/mg of protein, 37°C, 30 min) before western blot analysis with Saf Mix antibodies. An aliquot of each lysates was analysed before PK digestion with Saf32 antibodies (B) or anti-Myc antibodies (C).
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