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Endocytic Intermediates Involved with the Intracellular Trafficking of a Fluorescent Cellular Prion Protein*

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We have investigated the intracellular traffic of PrPc, a glycosylphosphatidylinositol (GPI)-anchored protein implicated in spongiform encephalopathies. A fluorescent functional green fluorescent protein (GFP)-tagged version of PrPc is found at the cell surface and in intracellular compartments in SN56 cells. Confocal microscopy and organelle-specific markers suggest that the protein is found in both the Golgi and the recycling endosomal compartment. Perturbation of endocytosis with a dynamin I-K44A dominant-negative mutant altered the steady-state distribution of the GFP-PrPc, leading to the accumulation of fluorescence in unfixed endocytic intermediates. These pre-endocytic intermediates did not seem to accumulate GFP-GPI, a minimum GPI-anchored protein, suggesting that PrPc trafficking does not depend solely on the GPI anchor. We found that internalized GFP-PrPc accumulates in Rab5-positive endosomes and that a Rab5 mutant alters the steady-state distribution of GFP-PrPc but not that of GFP-GPI between the plasma membrane and early endosomes. Therefore, we conclude that PrPc internalizes via a dynamin-dependent endocytic pathway and that the protein is targeted to the recycling endosomal compartment via Rab5-positive early endosomes. These observations indicate that traffic of GFP-PrPc is not determined predominantly by the GPI anchor and that, different from other GPI-anchored proteins, PrPc is delivered to classic endosomes after internalization.

The cellular prion protein (PrPc)1 is a glycosylphosphatidylinositol (GPI)-plasma membrane-anchored protein whose function is still under debate. Potential roles of PrPc in signaling events (1–5), cell adhesion and differentiation (6, 7), protection against oxidative insults (8–10), and copper metabolism (8, 11) have been suggested. Conversion of PrPc from an α-helix to a β-sheet-rich structure causes relevant biophysical changes to the protein that have been related to brain dysfunction in prion diseases (12–14). The mechanisms involved in this conversion are unknown, but accumulating evidence suggests that the process occurs after PrPc reaches the plasma membrane, and it may involve PrPc entry into intracellular acidic organelles (15–17).

The mechanisms of PrPc trafficking are poorly understood. A chicken PrPc has been shown to cycle through the plasma membrane and endosomes (18), and this process has been suggested to involve clathrin-mediated endocytosis (19). However, other evidence suggests that mammalian PrPc may follow a similar endocytic pathway as that of most GPI-anchored proteins. In particular, PrPc can be found in lipid rafts at the plasma membrane that are isolated as detergent-insoluble glycolipid vesicles (17, 20–22). Moreover, it has been suggested that internalization of PrPc occurs via a clathrin-independent mechanism, probably through "caveolae" (20, 21).

Internalization of GPI-anchored proteins is a complicated cellular event, because these proteins lack intracellular oriented sequences that are relevant for interaction with endocytic adaptor proteins. A minimum fluorescent GPI-anchored protein, GFP (green fluorescent protein)-GPI continuously cycles through the plasma membrane and the Golgi compartment without passing through classic endocytic organelles in a clathrin-independent fashion, suggesting that there is a default trafficking pathway that is followed by some GPI-anchored proteins (23). However, other GPI-anchored proteins such as the folate receptor pass through recycling endosomes before returning to the plasma membrane (23–25). Lipid rafts are heterologous structures and not all GPI-anchored proteins are clustered in the same rafts in cells (22). Thus, it is possible that multiple endocytic pathways contribute to the internalization of different GPI proteins.

Recently, we (26) and others (27–29) have generated distinct fluorescent PrPc molecules (GFP-PrPc). The fluorescent protein

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1 The abbreviations used are: PrPc, cellular prion protein; GPI, glycosylphosphatidylinositol; GFP, green fluorescent protein; MEM, minimal essential medium; DIC, differential interference contrast; Tfn, transferrin.
is correctly targeted to the plasma membrane, where it is anchored by GPI (26, 28) and is present in rafts (28). Importantly, copper induces GFP-PrPc internalization (26) in a similar way to its effect on PrPc (11, 30), suggesting that the fluorescent protein is functional and can be used to infer PrPc traffic in living cells.

In the present work we examined the intracellular localization of GFP-PrPc and disrupted distinct steps of the endocytic pathway to uncover intermediates involved in PrPc trafficking. Moreover, we tested whether the same endocytic intermediates required for PrPc trafficking also participate in the trafficking of a minimum GPI-anchored protein, GFP-GPI. We found that the steady-state distribution of GFP-PrPc is dynamin-regulated and that internalized GFP-PrPc is localized to Rab5-positive early endocytic vesicles and endosomes. In contrast, GFP-GPI is not found in the same endocytic organelles as GFP-PrPc. Consequently, we suggest that PrPc trafficking differs from that of other standard GPI-anchored proteins and may depend on additional internalization signals present in the protein.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The SN56 cells were a generous gift from Prof. Bruce Wainer (Department of Pathology, Emory University School of Medicine, Atlanta, GA). Cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma Chemical Co., St. Louis, MO), 10% fetal bovine serum (Invitrogen), 2 mM l-glutamine, and 1% penicillin/streptomycin in 25-cm² culture bottles in a 5% CO₂ atmosphere at 37 °C as described previously (26, 32). The SN56 cells were derived from septum neurons (31) and present a number of neuronal features, including expression of synaptic vesicle proteins (32) and neuronal type calcium channels (33). Such features are increased by differentiation (33, 34).

**Plasmids**—The GFP-PrPc vector has been described previously (26). The constitutively activated Rab5 mutant Q79L (Q79L), dynamin I (dynamin), and the dominant-negative dynamin I mutant K44A (K44A) plasmids were a gift from Prof. Marc G. Caron (Department of Cell Biology, Duke University and Howard Hughes Medical Institute). GFP-GPI was a gift from Benjamin J. Nichols and J. Lippincott-Schwartz (Medical Research Council Laboratory of Molecular Biology, United Kingdom, and Cell Biology and Metabolism Branch, NICHD, National Institutes of Health).

**Cell Transfection**—The SN56 cells were plated on coverslips 1 day before transfection. Cell transfection was performed by the liposome-mediated method (LipofectAMINE 2000, Invitrogen, Gaithersburg, MD) according to the manufacturer’s instructions. One microgram of plasmid and 2.5 μL of LipofectAMINE 2000 were used for 5.5 × 10⁶ cells. After 4 h of transfection, cells were maintained in serum-free medium and differentiated for 2 days. In co-transfection experiments we used 3–4 μg of DNA (with a proportional change of LipofectAMINE 2000), following a plasmid ratio of 1:3 (Rab5-Q79L) or 1:2 (dynamin I or dynamin I-K44A) for GFP-PrPc and the other plasmids.
Fluorescence Imaging—Live cell experiments were performed at room temperature (20–25 °C). Cells on coverslips were washed in MEM (minimal essential medium, without phenol red) and transferred to a custom holder in which the coverslip formed the bottom of a 400-μl bath. Imaging was performed with a Bio-Rad MRC 1024 laser scanning confocal system running the software Lasersharp 3.0 coupled to a Zeiss microscope (Axiovert 100) with a water immersion objective (40×, 1.2 numerical aperture) as described previously (26). Image analysis and processing were performed with Lasersharp (Bio-Rad), Confocal Assistant, Adobe Photoshop, and Metamorph (Universal Imaging) software.

PrP Internalization Assay—This assay has been previously described and shown to be dependent on the presence of the intact octarepeat Cu²⁺ binding region of PrP⁎ (26). Cells were perfused with MEM and, after obtaining the first Z series (0 min), MEM with or without 250–500 μM Cu²⁺ was perfused and another Z series was acquired (15 min).

Labeling of Organelles—To label endocytic organelles, we used the styryl dye FM4-64 (Molecular Probes, Eugene, OR). Cells were incubated with 16 μM FM4-64 for 15–40 min at 37 °C in 5% CO₂, and then visualized by confocal microscopy as previously described (35). Labeling of endosomes was performed by incubating cells with 40 μg/ml Alexa Fluor 568-labeled transferrin (Tfn-568, Molecular Probes) at 37 °C in 5% CO₂ for 20 min. After incubation, cells were washed three times with ice-cold phosphate-buffered saline and then either imaged or fixed.
with 3% paraformaldehyde in phosphate-buffered saline for 20 min for posterior imaging. Golgi complex was identified with Ceramide-Bodipy TR (Molecular Probes) as follows: cells were washed in HEPES-buffered salt solution (in millimolar: 137 NaCl, 4 KCl, 2 CaCl₂, 1.2 MgSO₄, 10 glucose, 10 HEPES, pH 7.4, adjusted with NaOH) and preincubated for 15 min at 37°C and then 15 min at 4°C with 5 μM Bodipy TR complexed with bovine serum albumin. Cells were then washed with HEPES-buffered salt solution and incubated for further 30 min at 37°C before imaging. Two types of Ceramide-Bodipy (FL and TR) labeled the same structures in SN56 cells, and labeling was completely abolished by Brefeldin A. Moreover, Ceramide-Bodipy FL, which fluoresces in green, co-localized with a red fluorescent variant of PrP⁺ (not shown).

RESULTS

Co-localization of GFP-PrP⁺ with Golgi and Endocytic Markers—Previous experiments using different GFP-PrP⁺ constructions have suggested that the fluorescent protein labels the Golgi compartment, as assessed by the co-localization of the GFP-tagged protein with a number of Golgi markers (27–29). Optical sections of living cells double-labeled with GFP-PrP⁺ and Ceramide-Bodipy TR confirmed these previous observations showing an excellent degree of localization of GFP-PrP⁺ in the Golgi apparatus in SN56 cells (Fig. 1, A–C). However, a significant proportion of GFP-PrP⁺ was localized to vesicular structures that were labeled by the endosomal compartment marker Tfn-568 (Fig. 1, D–F). A large proportion of the double-labeled endosomes were packed within the perinuclear region, although rare puncta in close proximity to the plasma membrane were also observed (Fig. 1D, arrow). Experiments with the vital dye FM4-64 showed that the GFP-PrP⁺-labeled perinuclear structure can be partially labeled with this endocytic tracer (Fig. 1, G–J). These observations suggest that intracellular GFP-PrP⁺ is accumulating not only in the Golgi but also in endosomal compartments.

Constitutive Traffic of GFP-PrP⁺ but Not of GFP-GPI Is Perturbed by Dynamin I K44A—To determine the initial steps involved with PrP⁺ trafficking, we examined whether the expression of a dynamin I K44A mutant might perturb GFP-PrP⁺ and GFP-GPI (a marker of non-clathrin-mediated endocytosis (23)) distribution in SN56 cells. Expression of the dynamin I-K44A dominant-negative mutant has been used extensively as a tool to block fission of endocytic intermediates (36). When dynamin I-K44A was co-expressed with GFP-PrP⁺ in SN56 cells, GFP-PrP⁺ was localized in structures close to the plasma membrane surface (Fig. 2, E–G). In contrast, in cells co-expressing wild-type dynamin I, labeling of these structures was not observed (Fig. 2, A–D). The internalization of FM4-64 (an endocytic tracer dye) was also decreased in dynamin I-K44A-expressing cells (Fig. 2, compare B with F), suggesting that most of the FM4-64 accumulation in the perinuclear region depends on dynamin activity. As expected, expression of dynamin I-K44A also inhibited by 75% the internalization of fluorescent transferrin by these cells, suggesting that this mutant potently inhibits clathrin-mediated endocytosis (37).

Fig. 2 (I–L) shows in more detail the GFP-PrP⁺ structures that appear in dynamin I-K44A co-transfected cells. Few vesicles labeled with GFP-PrP⁺ close to the plasma membrane could also be labeled with FM4-64 (Fig. 2, I–L). The labeling varied from cell to cell, but some cells showed large numbers of GFP-positive structures that were also stained with FM4-64 (see Fig. 2J). Optical sectioning of regions close to the top of the cell shows that these structures most often appeared to be connected to the plasma membrane and perhaps to the exterior milieu, because they are accessible to the impermeant dye FM4-64 (Fig. 2, I and J). Sections toward the middle of the cell show the presence of fluorescent GFP puncta in close association with the plasma membrane (Fig. 2, K and L). The structures labeled with GFP-PrP⁺ in dynamin I-K44A-expressing cells were similar to those described for some plasma membrane receptors that continuously traffic between the plasma membrane and the cytoplasm (38). In some cells (Fig. 2E), the expression of the GFP-PrP⁺ was limited to intense vesicular patches with very little diffuse labeling of the plasma membrane, suggesting that the GFP-PrP⁺ is constitutively endocytosed. In contrast, under conditions where expression of the dominant-negative dynamin I-K44A mutant blocked uptake of FM4-64 into SN56 cells, there is little change in the subcellular localization of GFP-GPI (compare Fig. 3, A and E). Thus, GFP-GPI exhibits a similar pattern of distribution in wild-type and mutant dynamin-expressing cells (Fig. 3). These observations...
indicate that the structures labeled with GFP-PrPc do not participate in the internalization of GFP-GPI.

**GFP-PrPc but Not GFP-GPI Is Trafficked to Rab5-positive Endosomes**—The observation that GFP-PrPc, but not GFP-GPI, accumulated in vesicles connected to the plasma membrane in the presence of dynamin I-K44A prompted us to investigate the cellular organelles underlying the constitutive trafficking of PrPc. We detected some events of co-localization between GFP-PrPc and Tfn-568 in puncta close to the plasma membrane in cells (Fig. 1), with a more extensive co-localization observed in the perinuclear region. Therefore, we examined whether GFP-PrPc either transits or bypasses the Rab5-positive early endosomal compartment to reach the perinuclear compartment of cells. Rab5 is involved in endosomal traffic and fusion. A Rab5-Q79L mutant that mimics the GTP-bound form of Rab5 and exhibits constitutive activity promotes homotypic fusion of endocytic vesicles into enlarged vesicular structures (39, 40). If GFP-PrPc bypasses this compartment, there should be no change in the subcellular distribution of the GFP-tagged protein in the presence of Rab5-Q79L.

Images of cells overexpressing Rab5-Q79L show that GFP-PrPc was readily identified in vesicles close to the plasma membrane (Fig. 4, A–C, green). Some of the GFP-PrPc-positive vesicles were also labeled with the vital dye FM4-64 (Fig. 4, A–C, superimposed images labeled with arrows), and in many cases we detected co-localization of GFP-PrPc with the endocytic marker Tfn-568 (Fig. 5, A and B). These results suggest the early endocytic origin of the GFP-PrPc-labeled vesicles. In agreement with a previous report (23), GFP-GPI did not localize in vesicles filled with FM4-64 or Tfn-568 in the presence of Rab5-Q79L (Figs. 4, D–F, and 5, C and D).

Our experiments suggest that constitutive traffic of GFP-PrPc is distinct from that of GFP-GPI for its sensitivity to K44A and presence in Rab5-positive endosomes. However, these studies did not determine whether GFP-PrPc enters early endosomes directly following internalization from the plasma membrane or whether the fluorescent protein is indirectly redistributed to earlier endosomes. It is known that copper binds to PrPc through an octarepeat region of amino acids (8, 41), and among other changes Cu²⁺/H₁₁₀₀₁ induces PrPc internalization (11, 26, 30). We thus used Cu²⁺ to evoke synchronized GFP-PrPc internalization in cells overexpressing Rab5-Q79L to determine whether these vesicles receive internalized GFP-PrPc. Fig. 6 (A and B) shows that GFP-PrPc responded to copper and accumulated in endocytic organelles in cells overexpressing Rab5-Q79L (arrows). In the absence of Rab5 Q79L, there was only limited accumulation of GFP-PrPc in early endocytic vesicles (26). However, short term exposure to Cu²⁺ in the absence of Rab5-Q79L induced the rapid accumulation of GFP-PrPc in the perinuclear compartment (Fig. 6, G and H) (26). Taken together, these observations suggest that under normal conditions GFP-PrPc transits very rapidly through the early endo-

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**Fig. 4.** GFP-PrPc co-localizes with endocytic vesicles away from the perinuclear region in Rab5-Q79L-expressing cells. SN56 cells were transfected with GFP-PrPc and the Rab5-Q79L mutant, or with GFP-GPI and Rab5-Q79L. Forty-eight hours after transfection, living cells were labeled with FM4-64 for 20 min and were examined by laser scanning confocal microscopy. Individual optical sections of a representative co-transfected cell expressing GFP-PrPc and Rab5-Q79L (green) and labeled with FM4-64 (red) are shown in A and B. Tfn-568 (red) was used as a marker for the endocytic vesicles. The result is representative for 90 cells examined with 70 cells presenting GFP-PrPc structures labeled with FM4-64. D and E, individual optical sections of a co-transfected cell expressing GFP-GPI and Rab5-Q79L (green) and labeled with FM4-64 (red). The Z axis distance between each image is shown at the bottom, and the Z projection of the same cell is presented in F. The result is representative of 34 cells in which only one showed co-localization of FM4-64 and GFP-GPI. Scale bar, 20 μm.
endocytosis (17, 19, 21). The localization of mammalian PrPc to Rab5-Q79L are shown in A and B, whereas two optical sections of a representative cell expressing GFP-GPI and Rab5-Q79L are shown in C and D. GFP-PrPc and GFP-GPI are presented in green, Tfn-568 is in red, and co-localization is seen in yellow in the superimposed images (lower panel). Arrows point to vesicles presenting co-localization of GFP-PrPc and Tfn-568. The Z axis distance between each image is shown at the bottom. The results are representative of 35 and 28 cells, respectively, for GFP-PrPc and GFP-GPI imaged in three independent experiments. Scale bar, 20 μm.

FIG. 5. GFP-PrPc co-localizes with Tfn-568 in Rab5 Q79L-expressing cells. SN56 cells were transfected with GFP-PrPc and the Rab5 Q79L mutant, or with GFP-GPI and Rab5-Q79L. Forty-eight hours after transfection, cells were labeled with Tfn-568 for 20 min, fixed with paraformaldehyde 3%, and examined by laser scanning confocal microscopy. Two optical sections of representative cells expressing GFP-PrPc and Rab5-Q79L are shown in A and B, whereas two optical sections of a representative cell expressing GFP-GPI and Rab5-Q79L are shown in C and D. GFP-PrPc and GFP-GPI are presented in green, Tfn-568 is in red, and co-localization is seen in yellow in the superimposed images (lower panel). Arrows point to vesicles presenting co-localization of GFP-PrPc and Tfn-568. The Z axis distance between each image is shown at the bottom. The results are representative of 35 and 28 cells, respectively, for GFP-PrPc and GFP-GPI imaged in three independent experiments. Scale bar, 20 μm.

Discussion

The present experiments using GFP-PrPc shed new light on the mechanisms underlying the internalization and trafficking of PrPc. We show that GFP-PrPc internalization is distinct from the non-clathrin-mediated endocytosis defined for GFP-GPI (23, 42), suggesting that PrPc-specific internalization signals exist and override the default pathway conferred by the GPI anchor. We also demonstrate that PrPc internalization is dependent upon dynamin I, a key mechano-enzyme involved with the fission of some, but not all (43, 44), endocytic vesicles from the plasma membrane. Moreover, we also identify Rab5-positive early endosomes as the initial destination of internalized GFP-PrPc in living cells.

Localization of PrPc in intracellular compartments has been reported in neurons (45–47), with strong expression in the Golgi (47). More recently, expression of GFP-PrPc by several research groups has also revealed the presence of the protein in the Golgi compartment (27–29). We have confirmed these data using a Golgi marker for living cells, however, by using endocytic markers and a Rab5 mutant we also found that part of the intracellular GFP-PrPc can be found in the endosomal compartment.

Discordant results have been reported regarding the mechanisms involved in the internalization of chicken and mammalian PrPc. Although chicken PrPc was reported to internalize via clathrin-dependent endocytic vesicles (18), mammalian PrPc was reported to depend on a caveolae-like mechanism of endocytosis (17, 19, 21). The localization of mammalian PrPc to classic endosomes is under debate (48), because many proteins found in lipid rafts are thought to bypass this compartment (23, 42, 49). In contrast, GPI-anchored proteins such as the folate receptor were found in Tfn-positive endosomes (24, 25). In the present study, we found that GFP-PrPc transited rapidly through the early endosomal compartment and that detection of GFP-PrPc in early endosomes was only observed when the compartment was perturbed by the overexpression of Rab5-Q79L. These results agree with recent ultrastructural localization of mammalian PrPc in neurons that has shown labeling of the Golgi but also of cytoplasmic vesicular organelles that might represent endosomes (47).

Internalization through caveoleae seems to divert proteins from the endosomal-lysosomal system (49). Moreover, caveoleae are immobile whenever cells are not stimulated, indicating that they may not participate in constitutive traffic events (50). Indeed, recent experiments suggest that non-clathrin-mediated endocytosis uses organelles distinct from classic endosomes (51). Internalization of GFP-GPI occurs independently of clathrin; the protein bypasses the early endosomal system and is not found in Rab5-Q79L generated endosomes (23, 51). If internalized GFP-PrPc was also capable of bypassing classic endosomes similar to GFP-GPI, it is unlikely that we would have found the fluorescent protein in Tfn-positive Rab5-Q79L-generated endosomes.

The presence of PrPc in caveolar-like organelles has been extensively reported (17, 20, 21), and an interaction between PrPc and caveolin has been suggested (3). The present data suggest that, if a non-clathrin mechanism of internalization of PrPc, such as caveoleae, is involved in the dynamin-dependent internalization of PrPc, it represents some sort of specialization that allows PrPc to enter the early endocytic pathway. An alternative view is that clathrin-coated vesicles are involved with mammalian PrPc trafficking similar to what has been described for chicken PrPc (19). In agreement with the later view, mammalian PrPc in neurons is found in coated pits (22, 47). These results agree with the observations that both intracellular injection of anti-caveolin or anti-clathrin antibodies inhibited activation of the Fin kinase (that presumably occurs intracellularly) induced by PrPc cross-linking with specific
antibodies (3). Together these results indicate that the initial recruitment of PrPc to pre-endocytic membranes is a complex event and may occur through more than one mechanism.

PrPc is a Cu²⁺ binding protein (8), but the consequences of Cu²⁺ interaction with the protein for its physiological function are poorly understood. It has been suggested that PrPc is used to deliver Cu²⁺ to intracellular compartments (11) or that the protein takes part in a mechanism of protection from oxidative insults (8–10). Coordinated decrease of fluorescence from the plasma membrane with the simultaneous increase of GFP-PrPc presence in the perinuclear compartment in response to Cu²⁺ has been previously reported (26). However, very few small vesicles filled with GFP-PrPc can be distinguished close to the plasma membrane after Cu²⁺ exposure in control cells. In Rab5-Q79L-expressing cells, we could observe endosomes filled with GFP-PrPc in response to Cu²⁺ (Fig. 6). This result suggests that at least part of both constitutive and Cu²⁺-evoked GFP-PrPc endocytosis and intracellular trafficking is mediated by early endosomes. Nonetheless, after internalization GFP-PrPc seems to preferentially accumulate in a perinuclear compartment in SN56 cells.

It has been shown that some GPI-anchored proteins are retained in the recycling endosomal compartment in a cholesterol and “raft”-dependent fashion, suggesting that lipid “rafts” may have important roles in sorting GPI-anchored proteins from endosomes (25, 52). It is perhaps physiologically relevant that PrPc stays longer and accumulates at the perinuclear endosomal compartment, because the latter has lower pH than the early endosomal compartment (53). PrPc binding to Cu²⁺ is pH-sensitive (54, 55) and the presence of PrPc in more acidic endosomes and its tendency to accumulate therein may allow for correct conditions for PrPc to release Cu²⁺ inside the cell. Alternatively, internalization of PrPc to endosomes may have the role of switching off potential signaling through PrPc (4, 5, 48).

PrPc has been shown to cycle constitutively through the plasma membrane and intracellular compartments (18). The mechanisms underlying PrPc internalization have remained obscure, in particular, the role of dynamin in the fission of vesicles bearing PrPc has not been previously tested. This is an important question, because many plasma membrane proteins are internalized by a dynamin-independent pathway (42–44).

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FIG. 6. Cu²⁺-induced internalization of GFP-PrPc to Rab5-Q79L vesicles. SN56 cells were transiently transfected with the GFP-PrPc, GFP-PrPc, and Rab5 Q79L mutant or GFP-GPI and Rab5-Q79L. Forty-eight hours after transfection, cells were assayed for PrPc internalization through perfusion with MEM plus Cu²⁺ (500 μM) for 15 min. Z series were acquired before and after the perfusion. The maximum Z projection of a co-transfected cell (representative of 31 independent experiments in which only three failed to detect internalization) expressing GFP-PrPc and Rab5-Q79L is shown in A (t = 0 min) and B (t = 15 min). Images were pseudocolored to facilitate the distinction of differences in fluorescence. Arrows point to vesicles showing an increase in fluorescence after Cu²⁺ perfusion, and arrowheads point to membrane regions with pronounced loss of fluorescence. D (0 min) and E (15 min) show a Z projection of a co-transfected cell expressing GFP-GPI and Rab5-Q79L before and after perfusion with Cu²⁺ (representative of 12 independent experiments). No significant differences were observed in GFP-GPI distribution after Cu²⁺ perfusion. G (0 min) and H (15 min) show an example of a cell expressing only GFP-PrPc before and after exposure to Cu²⁺. Respective DIC images are presented in the right panels. Scale bar, 20 μm.
localization is dependent upon dynamin activity. We found that a number of endocytic intermediates are generated in the presence of dynamin-I-K44A that are with GFP-PrP\(^{\text{c}}\). These endocytic intermediates are localized in close proximity to the plasma membrane and can be labeled with the membrane impermeant dye FM4-64, suggesting that the lumen of these intermediates is in contact with the extracellular medium. These results are in sharp contrast with those obtained with GFP-GPI, whose localization seems undisturbed in the presence of dynamin-I-K44A.

There are reports of proteins that interact with PrP\(^{\text{c}}\) with the potential to regulate internalization (4, 58, 59). Whether these or other proteins may participate in selection of PrP\(^{\text{c}}\) for internalization or are responsible for diverting the fluorescent protein to the early endosomal pathway in dynamin I-generated vesicles is at present unknown.

In conclusion, our data confirm that GPI-anchored proteins may follow distinct pathways in cells and show that constitutive trafficking of PrP\(^{\text{c}}\) differs in at least two fundamental aspects from the trafficking of GFP-GPI. First, we observed that the intracellular trafficking of cell surface PrP\(^{\text{c}}\) involves a dynamin-sensitive step. Second, we show that at least part of the internalized PrP\(^{\text{c}}\) transits through the Rab5-regulated early endosomal compartment prior to the accumulation of PrP\(^{\text{c}}\) in the perinuclear compartment of cells. We propose that the dynamin-dependent endocytosis of PrP\(^{\text{c}}\) is GPI anchor-independent and may be mediated by the interaction of proteins with other domains of PrP\(^{\text{c}}\).

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