9-6-2002

Endocytic intermediates involved with the intracellular trafficking of a fluorescent cellular prion protein

AC Magalhaes

JA Silva

VR Martins

VF Prado
Western University

SSG Ferguson

See next page for additional authors

Authors
AC Magalhaes, JA Silva, VR Martins, VF Prado, SSG Ferguson, MV Gomez, RR Brentani, and MAM Prado

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 36, Issue of September 6, pp. 33311–33318, 2002
Printed in U.S.A.

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

Received for publication, April 16, 2002, and in revised form, June 11, 2002
Published, JBC Papers in Press, June 17, 2002, DOI 10.1074/jbc.M203661200

Ana C. Magalhães,[a,b,c] Juliana A. Silva,[a,b,d] Kil S. Lee,[e,f,g] Vilma R. Martins,[e] Vania F. Prado,[h] Stephen S. G. Ferguson,[i] Marcus V. Gomez,[a] Ricardo R. Brentani,[e] and Marco A. M. Prado[a,j]

From the [a]Laboratório de Neurofarmacologia, Departamento de Farmacologia and the [h]Departamento de Bioquímica-Imunologia, ICB, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais 31270-910, Brazil, the [e]Ludwig Institute for Cancer Research, São Paulo Branch, Rua Antonio Prudente 109/4A, São Paulo 01509-010 SP, Brazil, the [f]Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo 01509-010, Brazil, and the [i]J. P. Robarts Research Institute and Department of Physiology, University of Western Ontario, 100 Perth Dr., P. O. Box 5015, London, Ontario N6A 5K8, Canada

We have investigated the intracellular traffic of PrP^c, a glycosylphosphatidylinositol (GPI)-anchored protein implicated in spongiform encephalopathies. A fluorescent functional green fluorescent protein (GFP)-tagged version of PrP^c 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-PrP^c, leading to the accumulation of fluorescence in unfissioned endocytic intermediates. These pre-endocytic intermediates did not seem to accumulate GFP-GPI, a minimum GPI-anchored protein, suggesting that PrP^c trafficking does not depend solely on the GPI anchor. We found that internalized GFP-PrP^c accumulates in Rab5-positive endosomes and that a Rab5 mutant alters the steady-state distribution of GFP-PrP^c but not that of GFP-GPI between the plasma membrane and early endosomes. Therefore, we conclude that PrP^c 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-PrP^c is not determined predominantly by the GPI anchor and that, different from other GPI-anchored proteins, PrP^c is delivered to classic endosomes after internalization.

The cellular prion protein (PrP^c)[1] is a glycosylphosphatidylinositol (GPI)-plasma membrane-anchored protein whose function is still under debate. Potential roles of PrP^c 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 PrP^c 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 PrP^c reaches the plasma membrane, and it may involve PrP^c entry into intracellular acidic organelles (15–17).

The mechanisms of PrP^c trafficking are poorly understood. A chicken PrP^c 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 PrP^c may follow a similar endocytic pathway as that of most GPI-anchored proteins. In particular, PrP^c 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 PrP^c 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 PrP^c molecules (GFP-PrP^c). The fluorescent protein

* This work was supported in part by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) Grant 99/07124-8 (to V. R. M.), Pronex, Programa de Apoio ao Desenvolvimento Científico e Tecnológico (PADCT), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG) (to A. M. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[b] Both authors contributed equally to this work.

[c] Received a Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) Ph.D. fellowship.
[d] Supported by a CNPq Post-doctoral fellowship.
[g] Received a Ph.D. fellowship from FAPESP (00/03629-7).

[j] To whom correspondence should be addressed: Laboratório de Neurofarmacologia, Departamento de Farmacologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antonio Carlos 6627, Belo Horizonte, Minas Gerais 31270-910, Brazil. Tel.: 55-31-3499-2718; Fax: 55-31-3499-2695; E-mail: mprado@icb.ufmg.br.

[1] The abbreviations used are: PrP^c, cellular prion protein; GPI, glycosylphosphatidylinositol; GFP, green fluorescent protein; MEM, minimal essential medium; DIC, differential interference contrast; Tfn, transferrin.

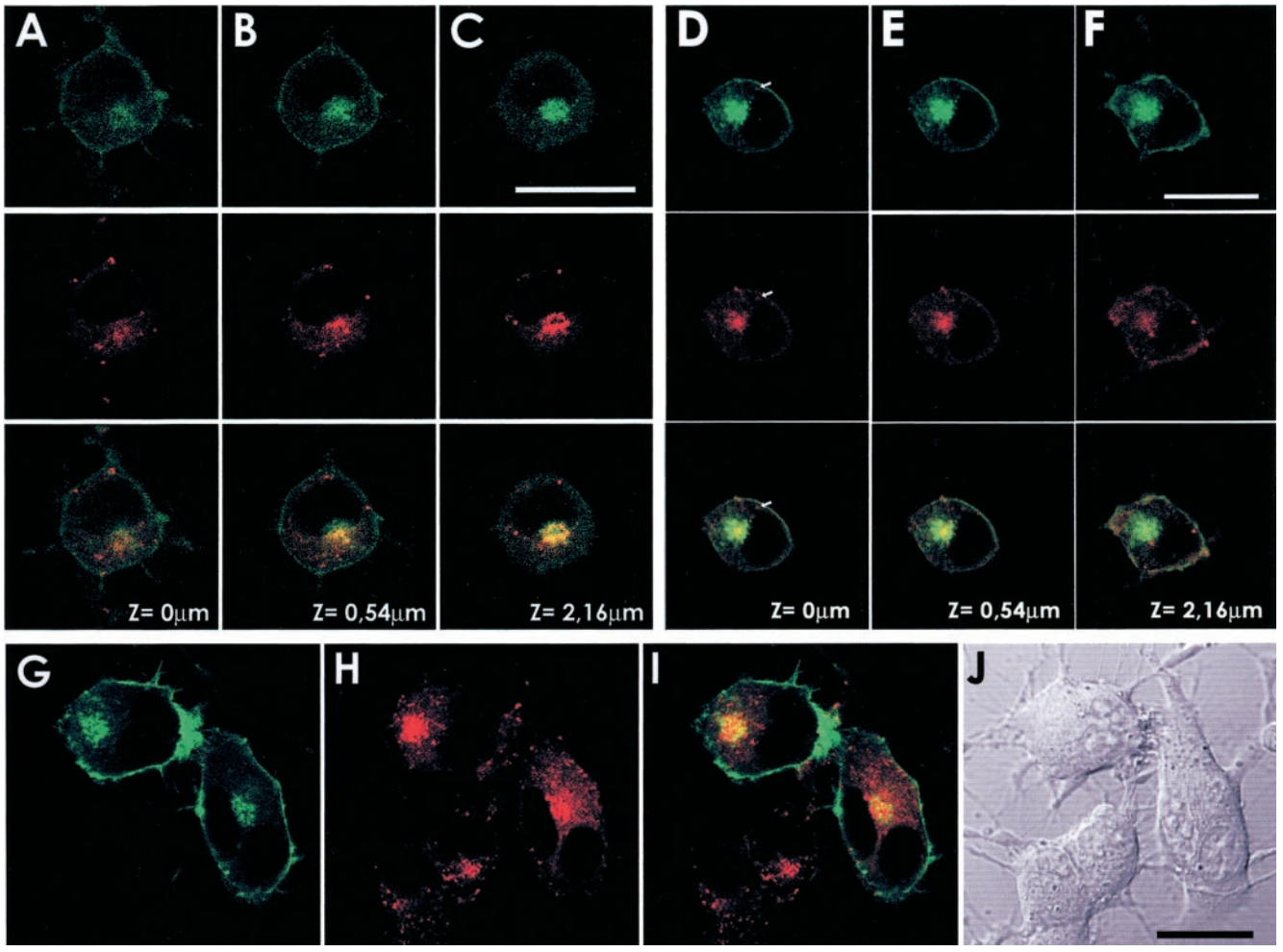

FIG. 1. **Colocalization of GFP-PrP^c with Golgi and endosomal markers.** SN56 cells were transiently transfected with the GFP-PrP^c construct and 48 h after transfection, living cells were labeled with the specified marker and examined by laser scanning confocal microscopy. *A–C*, individual optical sections of the same cell transfected with GFP-PrP^c (*green*, *upper panel*) and labeled with Ceramide-Bodipy TR (*red*, *middle panel*) and the respective superimposed images (*lower panel*). The distance of the Z axis between each image is shown. The cell is representative of 59 cells examined in different experiments. *D–F*, individual optical sections of a cell transfected with GFP-PrP^c (*green*, *upper panel*) and labeled with Transferrin-alexa 568 (*red*, *middle panel*) and the respective superimposed images (*lower panel*). The distance of the Z axis between each image is shown. The cell is representative of 23 cells examined in different experiments. *G*, an optical section of SN56 cells transfected with GFP-PrP^c; *H*, an optical section of SN56 cells labeled with FM4-64. *I*, overlay of *G* and *H*. *J*, respective differential interference contrast (DIC) image. The cells are representative of 37 cells examined. *Scale bars*, 20 μm.

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-PrP^c internalization (26) in a similar way to its effect on PrP^c (11, 30), suggesting that the fluorescent protein is functional and can be used to infer PrP^c traffic in living cells.

In the present work we examined the intracellular localization of GFP-PrP^c and disrupted distinct steps of the endocytic pathway to uncover intermediates involved in PrP^c trafficking. Moreover, we tested whether the same endocytic intermediates required for PrP^c trafficking also participate in the trafficking of a minimum GPI-anchored protein, GFP-GPI. We found that the steady-state distribution of GFP-PrP^c is dynamin-regulated and that internalized GFP-PrP^c is localized to Rab5-positive early endocytic vesicles and endosomes. In contrast, GFP-GPI is not found in the same endocytic organelles as GFP-PrP^c. Consequently, we suggest that PrP^c 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-cm^2 culture bottles in a 5% CO_2 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-PrP^c 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 5.5×10^4 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-PrP^c and the other plasmids.

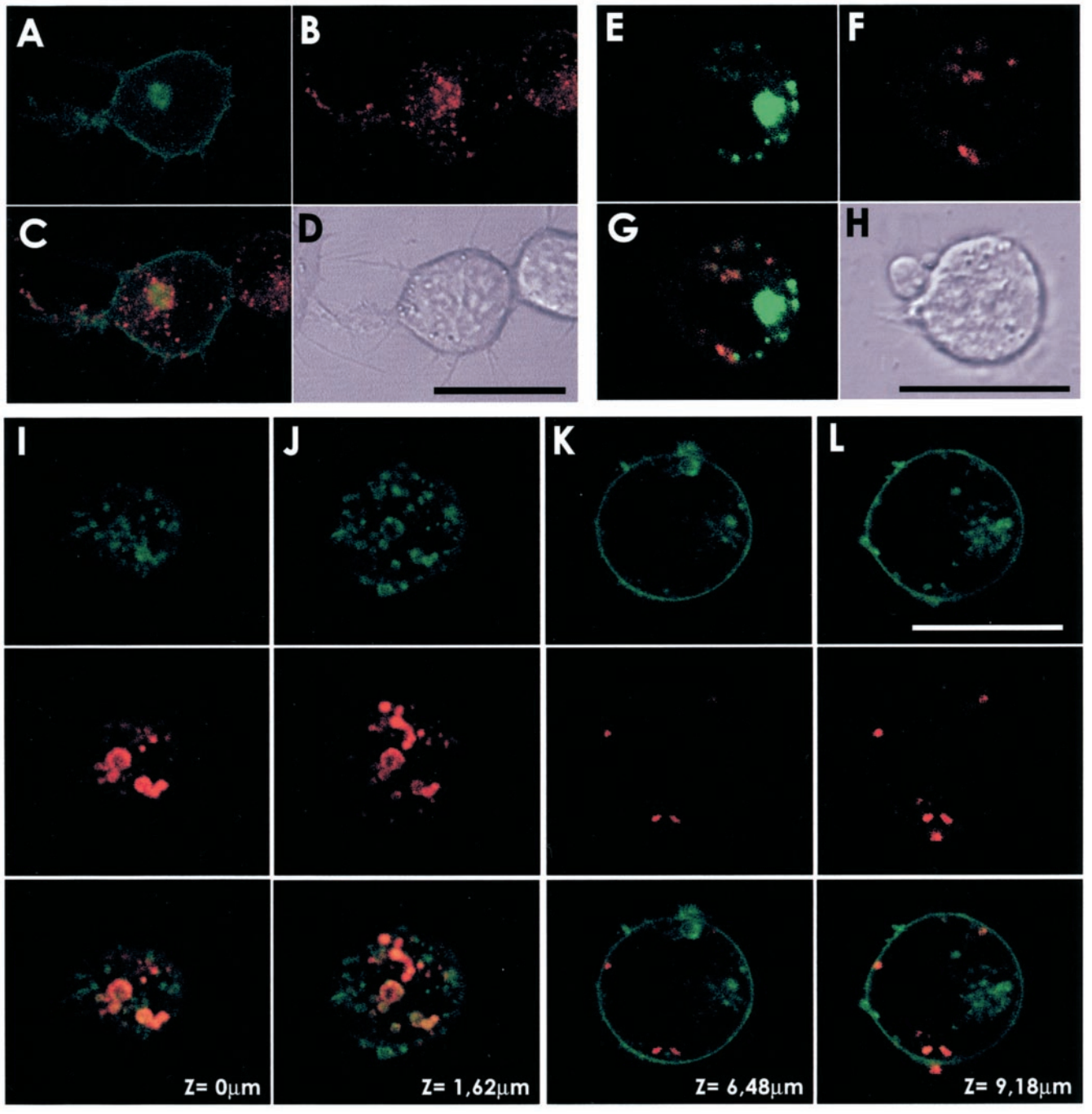

FIG. 2. **Dynamin is involved in PrP^c endocytosis.** SN56 cells were co-transfected with construct GFP-PrP^c and Dynamin or the mutant K44A. Forty-eight hours after transfection, living cells were labeled with FM4-64 and examined by laser scanning confocal microscopy. *A*, digital reconstruction (maximum Z projection) of a cell co-transfected with GFP-PrP^c and Dynamin. Note that FM4-64 can be internalized by this cell (*B*). *C*, shows superimposed images and (*D*) the respective DIC image. *E*, maximum Z projection of a cell co-transfected with GFP-PrP^c and the mutant K44A. Note the fluorescent structures close to the plasmalemma. *F*, internalization of FM4-64 for the same cell. Dynamin I-K44A-transfected cells internalized much less FM4-64 than Dynamin-transfected cells. *G*, overlay of images *E* and *F*. *H*, respective DIC image. *I–L*, individual optical sections of a cell co-transfected with GFP-PrP^c and Dynamin I-K44A (*green*, *upper panel*) and labeled with FM4-64 (*red*, *middle panel*) and the respective superimposed images (*lower panel*). The distance at the Z axis between each image is shown. *Scale bar*, 20 μm.

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^c Internalization Assay—This assay has been previously described and shown to be dependent on the presence of the intact octarepeat Cu^{2+} binding region of PrP^c (26). Cells were perfused with MEM and, after obtaining the first Z series (0 min), MEM with or without 250–500 μM Cu^{2+} 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% CO_2 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% CO_2 for 20 min. After incubation, cells were washed three times with ice-cold phosphate-buffered saline and then either imaged or fixed

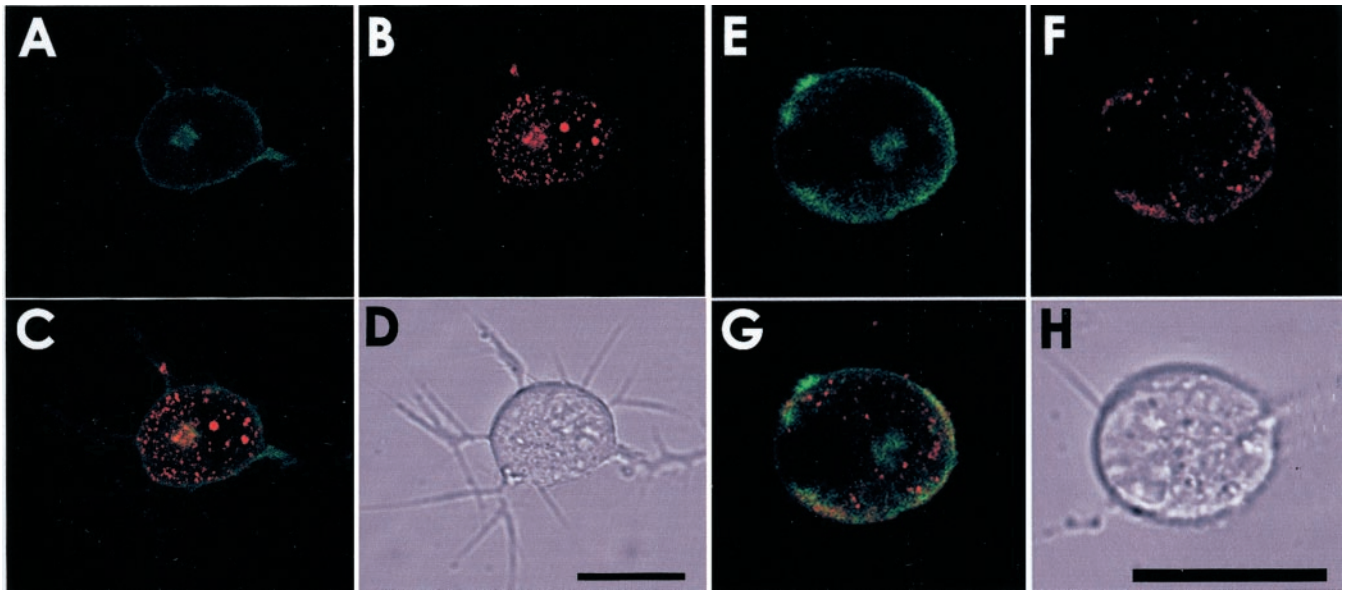

FIG. 3. **Dynamin I does not interfere with the distribution of GFP-GPI.** SN56 cells were co-transfected with the construct GFP-GPI and Dynamin (*A–D*) or the dynamin I mutant K44A (*E–H*). Forty-eight hours after transfection, living cells were labeled with FM4-64 and examined by laser scanning confocal microscopy. *A*, digital reconstruction of a cell co-transfected with GFP-GPI and Dynamin. *B*, internalization of FM4-64 by the same cell. *C*, shows the superimposed image and (*D*) the respective DIC image. *E*, digital reconstruction of a cell co-transfected with GFP-GPI and K44A. *F*, internalization of FM4-64 by the same cell, note that less dye is internalized and there is no perinuclear accumulation. *G*, shows the superimposed image and (*H*) the respective DIC image. *Scale bar*, 20 μm.

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 $CaCl_2$, 1.2 $MgSO_4$, 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^c (not shown).

RESULTS

Co-localization of GFP-PrP^c with Golgi and Endocytic Markers—Previous experiments using different GFP-PrP^c 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^c and Ceramide-Bodipy TR confirmed these previous observations showing an excellent degree of localization of GFP-PrP^c in the Golgi apparatus in SN56 cells (Fig. 1, *A–C*). However, a significant proportion of GFP-PrP^c 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. 1*D*, *arrow*). Experiments with the vital dye FM4-64 showed that the GFP-PrP^c-labeled perinuclear structure can be partially labeled with this endocytic tracer (Fig. 1, *G–J*). These observations suggest that intracellular GFP-PrP^c is accumulating not only in the Golgi but also in endosomal compartments.

Constitutive Traffic of GFP-PrP^c but Not of GFP-GPI Is Perturbed by Dynamin I K44A—To determine the initial steps involved with PrP^c trafficking, we examined whether the expression of a dynamin I-K44A mutant might perturb GFP-PrP^c 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^c in SN56 cells, GFP-PrP^c 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^c structures that appear in dynamin I-K44A co-transfected cells. Few vesicles labeled with GFP-PrP^c 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. 2*J*). Optical sectioning of regions close to the top of the cell shows that these structures most often appeared to be connected to the plasmalemma 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^c in dynamin I-K44A-expressing cells were similar to those described for some plasma membrane receptors that continuously traffic between the plasmalemma and the cytoplasm (38). In some cells (Fig. 2*E*), the expression of the GFP-PrP^c was limited to intense vesicular patches with very little diffuse labeling of the plasma membrane, suggesting that the GFP-PrP^c 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

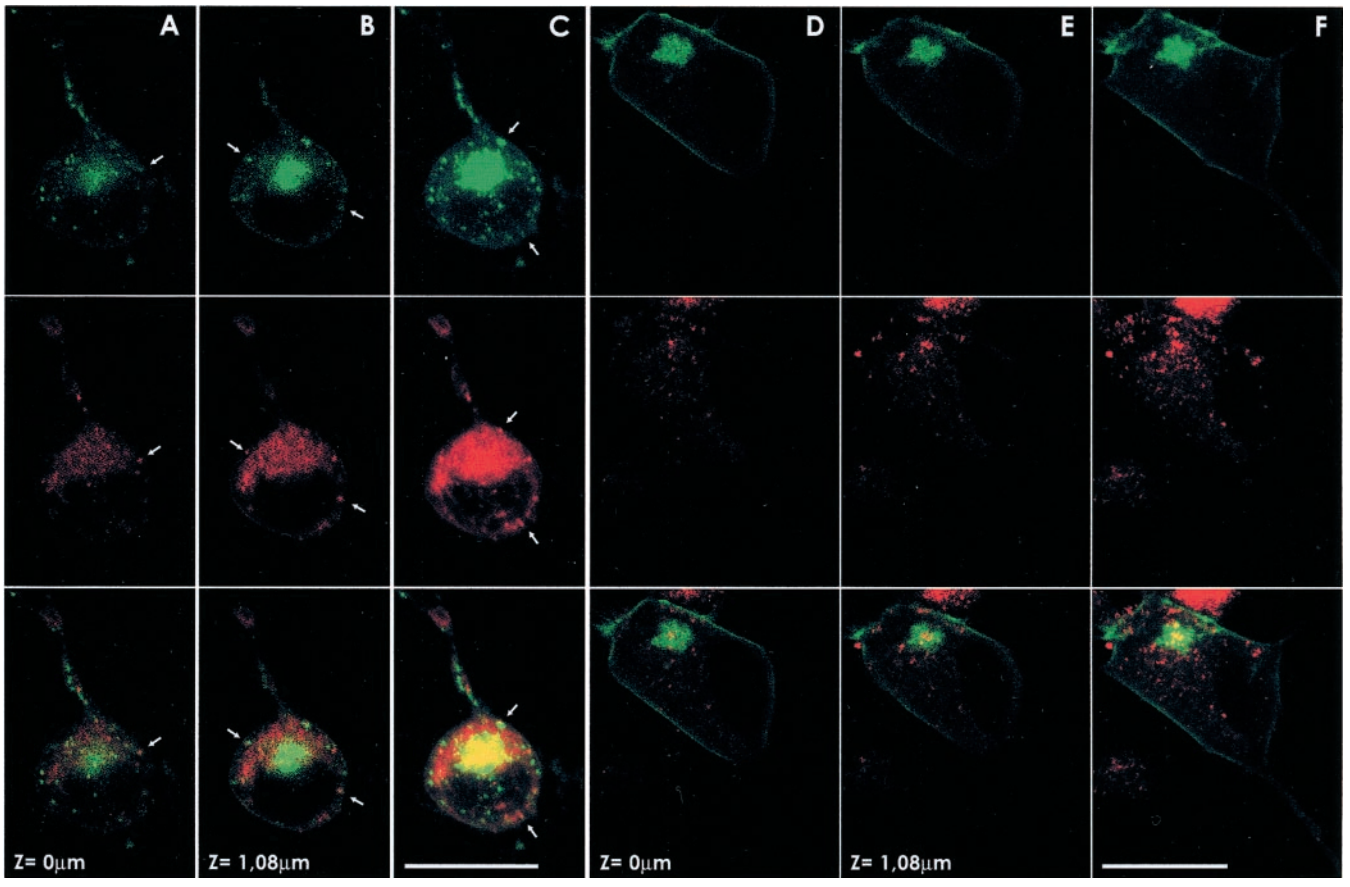

FIG. 4. **GFP-PrP^c co-localizes with endocytic vesicles away from the perinuclear region in Rab5-Q79L-expressing cells.** SN56 cells were transfected with GFP-PrP^c and the Rab5-Q79L mutant, or with GFP-GPI and Rab5-Q79L. Forty-eight hours after transfection, living cells were labeled with FM4-64 for 20 min and were examined by laser scanning confocal microscopy. Individual optical sections of a representative co-transfected cell expressing GFP-PrP^c and Rab5-Q79L (*green*) and labeled with FM4-64 (*red*) are shown in *A* and *B*. The Z axis distance between each image is shown at the *bottom*, and Z projection for this cell is presented in *C*. Note the presence of GFP-PrP^c vesicles away from the perinuclear region, which are uncommon in the absence of Rab5-Q79L construct (see Fig. 1). *Arrows* point to vesicles where co-localization is found. The result is representative for 90 cells examined with 70 cells presenting GFP-PrP^c 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.

indicate that the structures labeled with GFP-PrP^c do not participate in the internalization of GFP-GPI.

GFP-PrP^c but Not GFP-GPI Is Trafficked to Rab5-positive Endosomes—The observation that GFP-PrP^c, 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 PrP^c. We detected some events of co-localization between GFP-PrP^c 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-PrP^c 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-PrP^c 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-PrP^c was readily identified in vesicles close to the plasma membrane (Fig. 4, *A–C*, *green*). Some of the GFP-PrP^c-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-PrP^c with the endocytic marker Tfn-568 (Fig. 5, *A* and *B*). These results suggest the early endocytic origin of the GFP-PrP^c-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-PrP^c 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-PrP^c 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 PrP^c through an octarepeat region of amino acids (8, 41), and among other changes Cu^{2+} induces PrP^c internalization (11, 26, 30). We thus used Cu^{2+} to evoke synchronized GFP-PrP^c internalization in cells overexpressing Rab5-Q79L to determine whether these vesicles receive internalized GFP-PrP^c. Fig. 6 (*A* and *B*) shows that GFP-PrP^c 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-PrP^c in early endocytic vesicles (26). However, short term exposure to Cu^{2+} in the absence of Rab5-Q79L induced the rapid accumulation of GFP-PrP^c in the perinuclear compartment (Fig. 6, *G* and *H*) (26). Taken together, these observations suggest that under normal conditions GFP-PrP^c transits very rapidly through the early endo-

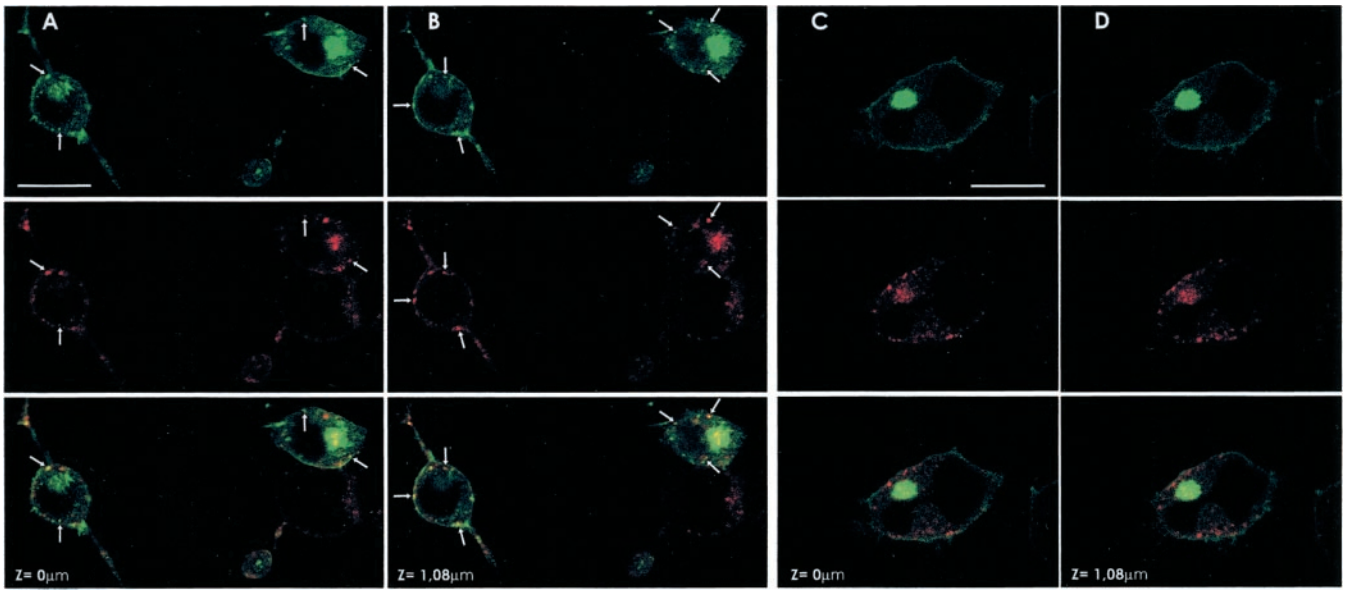

FIG. 5. **GFP-PrP^c co-localizes with Tfn-568 in Rab5 Q79L-expressing cells.** SN56 cells were transfected with GFP-PrP^c and the Rab5 Q79L mutant, or with GFP-GPI and Rab5-Q79L. Forty-eight hours after transfection, cells were labeled with Tfn-568 for 20 min, fixed with paraformaldehyde 3%, and examined by laser scanning confocal microscopy. Two optical sections of representative cells expressing GFP-PrP^c 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-PrP^c 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-PrP^c 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-PrP^c and GFP-GPI imaged in three independent experiments. *Scale bar*, 20 μm.

somal compartment and that only following the perturbation of endosomal trafficking is GFP-PrP^c detected in early endosomes. This may be the consequence of the detection limits for GFP-PrP^c, and its localization may require increased accumulation of protein in early endosomes following Rab5-Q79L expression. As expected, GFP-GPI, which is not found in Tfn-positive endosomes (23), did not become internalized through exposure of cells to Cu^{2+} (Fig. 6, *D* and *E*).

DISCUSSION

The present experiments using GFP-PrP^c shed new light on the mechanisms underlying the internalization and trafficking of PrP^c. We show that GFP-PrP^c internalization is distinct from the non-clathrin-mediated endocytosis defined for GFP-GPI (23, 42), suggesting that PrP^c-specific internalization signals exist and override the default pathway conferred by the GPI anchor. We also demonstrate that PrP^c 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-PrP^c in living cells.

Localization of PrP^c in intracellular compartments has been reported in neurons (45–47), with strong expression in the Golgi (47). More recently, expression of GFP-PrP^c 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-PrP^c can be found in the endosomal compartment.

Discordant results have been reported regarding the mechanisms involved in the internalization of chicken and mammalian PrP^c. Although chicken PrP^c was reported to internalize via clathrin-dependent endocytic vesicles (18), mammalian PrP^c was reported to depend on a caveolae-like mechanism of endocytosis (17, 19, 21). The localization of mammalian PrP^c 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-PrP^c transited rapidly through the early endosomal compartment and that detection of GFP-PrP^c 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 PrP^c in neurons that has shown labeling of the Golgi but also of cytoplasmic vesicular organelles that might represent endosomes (47).

Internalization through caveolae seems to divert proteins from the endosomal-lysosomal system (49). Moreover, caveolae 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-PrP^c 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 PrP^c in caveolar-like organelles has been extensively reported (17, 20, 21), and an interaction between PrP^c and caveolin has been suggested (3). The present data suggest that, if a non-clathrin mechanism of internalization of PrP^c, such as caveolae, is involved in the dynamin-dependent internalization of PrP^c, it represents some sort of specialization that allows PrP^c to enter the early endocytic pathway. An alternative view is that clathrin-coated vesicles are involved with mammalian PrP^c trafficking similar to what has been described for chicken PrP^c (19). In agreement with the later view, mammalian PrP^c 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 PrP^c cross-linking with specific

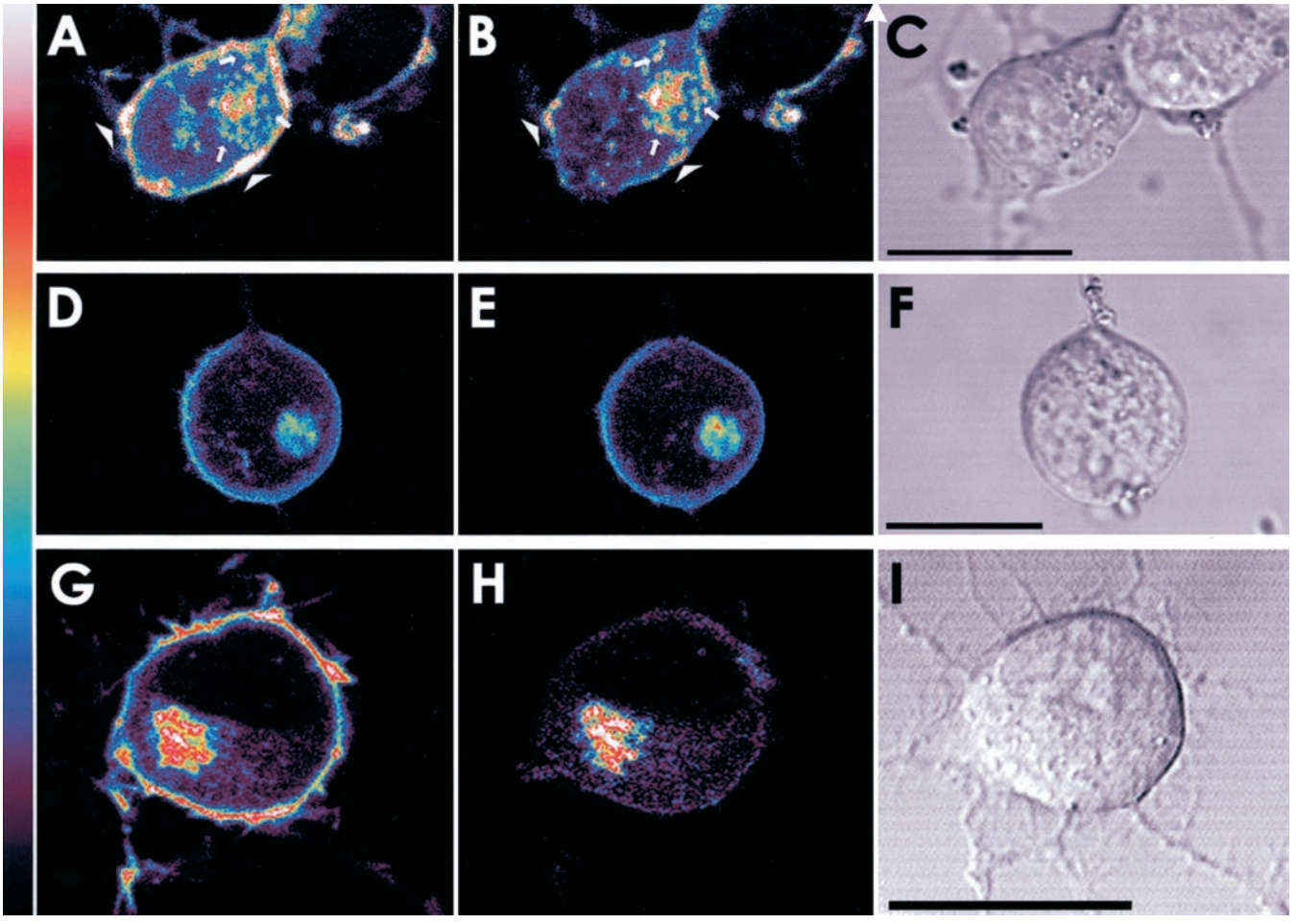

FIG. 6. **Cu^{2+}-induced internalization of GFP-PrP^c to Rab5-Q79L vesicles.** SN56 cells were transiently transfected with the GFP-PrP^c, GFP-PrP^c, and Rab5 Q79L mutant or GFP-GPI and Rab5-Q79L. Forty-eight hours after transfection, cells were assayed for PrP^c internalization through perfusion with MEM plus Cu^{2+} (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-PrP^c 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 Cu^{2+} 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 Cu^{2+} (representative of 12 independent experiments). No significant differences were observed in GFP-GPI distribution after Cu^{2+} perfusion. *G* (0 min) and *H* (15 min) show an example of a cell expressing only GFP-PrP^c before and after exposure to Cu^{2+}. Respective DIC images are presented in the *right panels*. *Scale bar*, 20 μm.

antibodies (3). Together these results indicate that the initial recruitment of PrP^c to pre-endocytic membranes is a complex event and may occur through more than one mechanism.

PrP^c is a Cu^{2+} binding protein (8), but the consequences of Cu^{2+} interaction with the protein for its physiological function are poorly understood. It has been suggested that PrP^c is used to deliver Cu^{2+} 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-PrP^c presence in the perinuclear compartment in response to Cu^{2+} has been previously reported (26). However, very few small vesicles filled with GFP-PrP^c can be distinguished close to the plasma membrane after Cu^{2+} exposure in control cells. In Rab5-Q79L-expressing cells, we could observe endosomes filled with GFP-PrP^c in response to Cu^{2+} (Fig. 6). This result suggests that at least part of both constitutive and Cu^{2+}-evoked GFP-PrP^c endocytosis and intracellular trafficking is mediated by early endosomes. Nonetheless, after internalization GFP-PrP^c 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 PrP^c stays longer and accumulates at the perinuclear endosomal compartment, because the latter has lower pH than the early endosomal compartment (53). PrP^c binding to Cu^{2+} is pH-sensitive (54, 55) and the presence of PrP^c in more acidic endosomes and its tendency to accumulate therein may allow for correct conditions for PrP^c to release Cu^{2+} inside the cell. Alternatively, internalization of PrP^c to endosomes may have the role of switching off potential signaling through PrP^c (4, 5, 48).

PrP^c has been shown to cycle constitutively through the plasma membrane and intracellular compartments (18). The mechanisms underlying PrP^c internalization have remained obscure, in particular, the role of dynamin in the fission of vesicles bearing PrP^c has not been previously tested. This is an important question, because many plasma membrane proteins are internalized by a dynamin-independent pathway (42–44). In fact, it is suggested that GPI-anchored proteins internalize via a dynamin-independent mechanism (56, 57). Our data obtained in cells overexpressing the dynamin I-K44A dominant-negative show that GFP-PrP^c trafficking and steady-state

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^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 are 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^c with the potential to regulate internalization (4, 58, 59). Whether these or other proteins may participate in selection of PrP^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^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^c involves a dynamin-sensitive step. Second, we show that at least part of the internalized PrP^c transits through the Rab5-regulated early endosomal compartment prior to the accumulation of PrP^c in the perinuclear compartment of cells. We propose that the dynamin-dependent endocytosis of PrP^c is GPI anchor-independent and may be mediated by the interaction of proteins with other domains of PrP^c.

Acknowledgments—We thank Drs. Bruce Wainer, Marc Caron, Ben Nichols, and Jennifer Lippincott-Schwartz for essential reagents used in this work. We also thank D. Abraão, A. Pereira, E. E. P. Silva, and C. Costa for technical assistance.

REFERENCES

1. Shmerling, D., Hegyi, I., Fischer, M., Blattler, T., Brandner, S., Gotz, J., Rulicke, T., Flechsig, E., Cozzio, A., von Mering, C., Hangartner, C., Aguzzi, A., and Weissmann, C. (1998) *Cell* **93,** 203–214
2. Herms, J. W., Korte, S., Gall, S., Schneider, I., Dunker, S., and Kretzschmar, H. A. (2000) *J. Neurochem.* **75,** 1487–1492
3. Mouillet-Richard, S., Ermonval, M., Chebassier, C., Laplanche, J. L., Lehmann, S., Launay, J. M., and Kellermann, O. (2000) *Science* **289,** 1925–1928
4. Zanata, S. M., Lopes, M. H., Mercadante, A. F., Hajj, G. N. M., Chiarini, L. B., Nomizo, R., Freitas A. R. O., Cabral, A. L. B., Lee, K. S., Juliano, M. A., Oliveira, E., Jachieri, S. G., Burlingame, A., Huang, L., Linden, R., Brentani, R. R., and Martins, V. R. (2002) *EMBO J.* **21,** 3307–3316.
5. Chiarini, L. B., Freitas, A. R. O., Zanata, S. M., Brentani, R. R., Martins, V. R., and Linden, R. (2002) *EMBO J.* **21,** 3317–3326
6. Graner, E., Mercadante, A. F., Zanata, S. M., Forlenza, O. V., Cabral, A. L., Veiga, S. S., Juliano, M. A., Roesler, R., Walz, R., Minetti, A., Izquierdo, I., Martins, V. R., and Brentani, R. R. (2000) *Brain. Res. Mol. Brain. Res.* **76,** 85–92
7. Graner, E., Mercadante, A. F., Zanata, S. M., Martins, V. R., Jay, D. G., and Brentani, R. R. (2000) *FEBS Lett.* **482,** 257–260
8. Brown, D. R., Qin, K., Herms, J. W., Madlung, A., Manson, J., Strome, R., Fraser, P. E., Kruck, T., von Bohlen, A., Schulz-Schaeffer, W., Giese, A., Westaway, D., and Kretzschmar, H. (1997) *Nature* **390,** 684–687
9. Brown, D. R. (2001) *Trends Neurosci.* **24,** 85–90
10. Klamt, F., Dal-Piolzz, F., Conte da Frota, M. L. J. R., Walz, R., Andrades, M. E., da Silva, E. G., Brentani, R. R., Izquierdo, I., and Fonseca Moreira, J. C. (2001) *Free Radic. Biol. Med.* **30**, 1137–1144
11. Pauly, P. C., and Harris, D. A. (1998) *J. Biol. Chem.* **273,** 33107–33110
12. Prusiner, S. B. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 13363–13383
13. Weissmann, C. (1999) *J. Biol. Chem.* **274,** 3–6
14. Aguzzi, A., Glatzel, M., Montrasio, F., Prinz, M., and Heppner, F. L. (2001) *Nat. Rev. Neurosci.* **2,** 745–749
15. Caughey, B., and Raymond, G. J. (1991) *J. Biol. Chem.* **266,** 18217–18223
16. Caughey, B., Raymond, G. J., Ernst, D., and Race, R. E. (1991) *J. Virol.* **65,** 6597–6603
17. Taraboulos, A., Scott, M., Semenov, A., Avrahami, D., Laszlo, L., Prusiner, S. B., and Avrahami, D. (1995) *J. Cell Biol.* **129,** 121–132
18. Shyng, S. L., Huber, M. T., and Harris, D. A. (1993) *J. Biol. Chem.* **268,** 15922–15928
19. Shyng, S. L., Heuser, J. E., and Harris, D. A. (1994) *J. Cell Biol.* **125,** 1239–1250
20. Vey, M., Pilkuhn, S., Wille, H., Nixon, R., DeArmond, S. J., Smart, E. J., Anderson, R. G., Taraboulos, A., and Prusiner, S. B. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 14945–14949
21. Kaneko, K., Vey, M., Scott, M., Pilkuhn, S., Cohen, F. E., and Prusiner, S. B. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 2333–2338
22. Madore, N., Smith, K. L., Graham, C. H., Jen, A., Brady, K., Hall, S., and Morris, R. (1999) *EMBO J.* **18,** 6917–6926
23. Nichols, B. J., Kenworthy, A. K., Polishchuk, R. S., Lodge, R., Roberts, T. H., Hirschberg, K., Phair, R. D., and Lippincott-Schwartz, J. (2001) *J. Cell Biol.* **153,** 529–542
24. Rijnboutt, S., Jansen, G., Posthuma, G., Hynes, J. B., Schornagel, J. H., Strous, G. J. (1996) *J. Cell Biol.* **132,** 35–47
25. Mayor, S., Sabharanjak, S., and Maxfield, F. R. (1998) *EMBO J.* **17,** 4626–4638
26. Lee, K. S., Magalhães, A. C., Zanata, S. M., Brentani, R. R., Martins, V. R., and Prado, M. A. (2001) *J. Neurochem.* **79,** 79–87
27. Ivanova, L., Barmada, S., Kummer, T., and Harris, D. A. (2001) *J. Biol. Chem.* **276,** 42409–42421
28. Negro, A., Ballarin, C., Bertoli, A., Massimino, M. L., and Sorgato, M. C. (2001) *Mol. Cell. Neurosci.* **17,** 521–538
29. Lorensz Windl, O., and Kretzschmar, H. A. (2002) *J. Biol. Chem.* **277,** 8508–8516
30. Sumudhu, W., Perera, S., and Hooper, N. M. (2001) *Curr. Biol.* **11,** 519–523
31. Hammond, D. N., Lee, H. J., Tonsgard, J. H., and Wainer, B. H. (1990) *Brain Res.* **512,** 190–200
32. Barbosa, J., Jr., Massensini, A. R., Santos, M. S., Meireles, S. I., Gomez, R. S., Gomez, M. V., Romano-Silva, M. A., Prado, V. F., and Prado, M. A. M. (1999) *J. Neurochem.* **73,** 1881–1893
33. Kushmerick, C., Romano-Silva, A., Gomez, M. V., and Prado, M. A. (2001) *Brain Res.* **916,** 199–210
34. Blusztajn, J. K., Venturini, A., Jackson, D. A., Lee, H. J., and Wainer, B. H. (1992) *J. Neurosci.* **12,** 793–799
35. Santos, M. S., Barbosa, J., Jr., Veloso, G. S., Ribeiro, F., Kushmerick, C., Gómez, M. V., Ferguson, S. S., Prado, V. F., and Prado, M. A. (2001) *J. Neurochem.* **78,** 1104–1113
36. Damke, H., Baba, T., Warnock, D. E., and Schmid, S. L. (1994) *J. Cell Biol.* **127,** 915–934
37. Barbosa, J., Jr., Ferreira, L. T., Martins-Silva, C., Santos, M. S., Torres, G. E., Caron, M. G., Gomez, M. V., Ferguson, S. S. G., Prado, M. A. M., and Prado, V. F. (2002) *J. Neurochem.,* in press.
38. Anborgh, P. H., Seachrist, J. L., Dale, L. B., and Ferguson, S. S. (2000) *Mol. Endocrinol.* **14,** 2040–2053
39. Stenmark, H., Parton, R. G., Steele-Mortimer, O., Lutcke, A., Gruenberg, J., and Zerial, M. (1994) *EMBO J.* **13,** 1287–1296
40. Nielsen, E., Severin, F., Backer, J. M., Hyman, A. A., and Zerial, M. (1999) *Nat. Cell Biol.* **1,** 376–382
41. Viles, J. H., Cohen, F. E., Prusiner, S. B., Goodin, D. B., Wright, P. E., and Dyson, H. J. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 2042–2047
42. Nichols, B. J., Kenworthy, A. K., Polishchuk, R. S., Lodge, R., Roberts, T. H., Hirschberg, K., Phair, R. D., and Lippincott-Schwartz, J. (2001) *J. Cell Biol.* **153,** 529–541
43. Zhang, J., Ferguson, S. S. G., Barak, L. S., Ménard, L., and Caron, M. G. (1996) *J. Biol. Chem.* **271,** 18302–18305
44. Vickery, R. G., and von Zastrow, M. (1999) *J. Cell Biol.* **144,** 31–43
45. DeArmond, S. J., Mobley, W. C., DeMott, D. L., Barry, R. A., Beckstead, J. H., and Prusiner, S. B. (1987) *Neurology* **37,** 1271–1280
46. Piccardo, P., Safar, J., Ceroni, M., Gajdusek, D. C., and Gibbs, C. J. (1990) *Neurology* **40,** 518–522
47. Laine, J., Marc, M. E, Sy, M. S., and Axelrad, H. (2001) *Eur. J. Neurosci.* **14,** 47–56
48. Martins, V. M., Linden, R., Prado, M. A. M., Walz, R., Sakamoto, A. C., Izquierdo, I., and Brentani, R. R. (2002) *FEBS Lett.* **512,** 25–28
49. Anderson, R. G. (1998) *Annu. Rev. Biochem.* **67,** 199–255
50. Thomsen, P., Roepstorff, K., Stahlhut, M., and van Deurs, B. (2002) *Mol. Biol. Cell* **13,** 238–250
51. Nichols, B. J. (2002) *Nat. Cell Biol.* **4,** 374–378
52. Chatterjee, S., Smith, E. R., Hanada, K., Stevens, V. L., and Mayor, S. (2001) *EMBO J.* **20,** 1583–1592
53. Yamashiro, D. J., and Maxfield, F. R. (1987) *J. Cell Biol.* **105,** 2713–2721
54. Whittal, R. M., Ball, H. L., Cohen, F. E., Burlingame, A. L., Prusiner, S. B., and Baldwin, M. A. (2000) *Protein Sci.* **9,** 332–343
55. Miura, T., Hori-i, A., Mototani, H., and Takeuchi, H. (1999) *Biochemistry* **38,** 11560–11569
56. Skretting, G., Torgersen, M. L., van Deurs, B., and Sandvig, K. (1999) *J. Cell Sci.* **112,** 3899–3909
57. Ricci, V., Galmiche, A., Doye, A., Necchi, V., Solcia, E., and Boquet, P. (2000) *Mol. Biol. Cell* **11,** 3897–3909
58. Martins, V. R., Graner, E., Garcia-Abreu, J., de Souza, S. J., Mercadante, A. F., Veiga, S. S., Zanata, S. M., Neto, V. M., and Brentani R. R. (1997) *Nat. Med.* **3,** 376–382
59. Gauczynski, S., Peyrin, J. M., Haik, S., Leucht, C., Hundt, C., Rieger, R., Krasemann, S., Deslys, J. P., Dormont, D., Lasmezas, C. I., and Weiss, S. (2001) *EMBO J.* **20,** 5863–5875