This is an author produced version of a paper published in Journal of Neurochemistry. This paper has been peer-reviewed but does not include the final publisher proof-corrections or journal pagination.

Citation for the published paper:
Cheng F, Lindqvist J, Haigh CL, Brown DR, Mani K.
"Copper-dependent co-internalization of the prion protein and glypican-1."
Journal of Neurochemistry, 2006, Vol: 98, Issue: 5, pp. 1445-57.
http://dx.doi.org/10.1111/j.1471-4159.2006.03981.x

Access to the published version may require journal subscription.
Published with permission from: Blackwell

Copper-Dependent Co-Internalization of the Prion Protein and Glypican-1

Fang Cheng[‡], Josefin Lindqvist[‡], Cathryn L. Haigh[#], David R. Brown[#], and Katrin Mani[‡,§§]

From the [‡]Department of Experimental Medical Science, Division of Neuroscience, Glycobiology group, Lund University, Biomedical Center C13, SE-221 84, Lund, Sweden, and [#]Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, United Kingdom

[§§]Address correspondence and reprints to Katrin Mani, Department of Experimental Medical Science, Division of Neuroscience, Glycobiology group, Lund University, Biomedical Center C13, SE-221 84, Lund, Sweden. Tel.: +46 46 222 4077. Fax.: +46 46 222 3128. E-mail: katrin.mani@med.lu.se

Abbreviations used: anMan, anhydromannose; Gpc-1, glypican-1; GPI, glycosylphosphatidylinositol; HS, heparan sulfate; GFP, green fluorescent protein; NO, nitric oxide; PrP^{C}, prion protein; PG, proteoglycan; SNO, S-nitroso group.

Heparan sulfate chains have been found to be associated with amyloid deposits in a number of diseases including transmissible spongiform encephalopathies. Diverse lines of evidence have linked proteoglycans and their glycosaminoglycan chains and especially heparan sulfate to the metabolism of the prion protein isoforms. Glypicans are a family of glycosylphosphatidylinositol-anchored, heparan sulfate-containing, cell-associated proteoglycans. Cysteines in glypican-1 can become nitrosylated by endogenously produced nitric oxide. When glypican-1 is exposed to a reducing agent, such as ascorbate, nitric oxide is released and autocatalyzes deaminative cleavage of heparan sulfate chains. These processes take place while glypican-1 recycles via a non-classical, caveolin associated pathway. We have previously demonstrated that prion protein provides Cu^{2+} ions required to nitrosylate thiol groups in the core protein of glypican-1. By using confocal immunofluorescence microscopy and immunomagnetic techniques, we now show that copper induces co-internalization of prion protein and glypican-1 from the cell surface to perinuclear compartments. We find that prion protein is controlling both the internalization of glypican-1 and its nitric oxide-dependent autoprocessing. Silencing of glypican-1 expression has no effect on copper-stimulated prion protein endocytosis but in cells expressing a prion protein construct lacking the copper-binding domain, internalization of glypican-1 is much reduced and autoprocessing is abrogated. We also demonstrate that heparan sulfate chains of glypican-1 are poorly degraded in prion null fibroblasts. Addition of Cu^{2+} ions, nitric oxide-donor and ascorbate or ectopic expression of prion protein restores heparan sulfate degradation. These results indicate that interaction between glypican-1 and Cu^{2+} loaded prion protein is required both for co-internalization and glypican-1 self-pruning.

Keywords: Prion, Glypican, Heparan sulfate, Nitric oxide, Copper

Running title: Prion induced glypican processing

Since the formulation of the prion-only hypothesis for the transmissible spongiform encephalopathies (for review, see Prusiner 1998) there have been spectacular advances. The function of the normal cellular prion protein has been a subject of considerable debate (Aguzzi and Polymenidou 2004). As the protein binds copper it is highly likely that its function is related to this property (Brown 2001). These possibilities include sequestration via internalization (Pauly and Harris 1998), copper uptake (Brown 1999) and antioxidant activity (Brown et al., 1999). Other activities unrelated to copper binding have also been proposed such as cell signalling and adhesion (Mouillet-Richard et al., 2000; Mange et al., 2002).

Prion protein (PrP^C) is anchored to cellular membranes via a C-terminal glycosylphosphatidylinositol (GPI) bridge and GPI-linked PrP^C associates with lipid rafts and caveolae (Vey et al., 1996). The N-terminal domain of PrP^C contains octapeptide repeat motifs encompassing five Cu^{2+} ion-binding sites (Brown et al., 1997; Viles et al., 1999; Qin et al., 2002).

How copper binding contributes to the function of PrP^C has remained elusive. Earlier studies had indicated that the N-terminal domain of PrP^C is essential for Cu^{2+} ion stimulated endocytosis via clathrin-coated pits and subsequent recycling to the cell surface via endocytic compartments (Shyng et al., 1995; Pauly and Harris, 1998). Furthermore, green fluorescent protein (GFP)-tagged PrP^C could be identified both at the cell surface and in an intracellular, perinuclear compartment of a neuronal cell-line (Lee et al., 2001). Transfer of GFP-PrP^C from the cell surface to the perinuclear compartment was induced by exposure to Cu^{2+} ions. N-terminal deletion mutants lacking the copper-binding sites were only found at the cell surface and were not internalized in the presence of Cu^{2+} ions. Other studies have indicated that Cu^{2+} ions stimulate redistribution of PrP^C to a subset of transferrin-containing early endosomes, also pointing to a clathrin-dependent endocytosis (Brown and Harris, 2003).

In contrast, subsequent studies, using N2a cells, have argued that mammalian PrP^C is internalized by one of the non-coated pit mechanisms characterized for raft-associated proteins (Marella et al., 2002; Kaneko et al., 1997). It has also been demonstrated that when protein A-gold particles were specifically bound to PrP^C on live CHO cells stably transfected with PrP^C, the complexes were internalized via caveolae and delivered to perinuclear vesicles, presumably caveosomes (Peters et al., 2003). As clustering of lipid raft components can trigger caveolar

endocytosis (Pelkman and Helenius, 2002; Nichols, 2003), it is possible that PrP^C, through interactions with other raft components, can by-pass the classical, clathrin-mediated pathway.

Previous studies had indicated that sulfated glycosaminoglycans, especially heparan sulfate (HS) are involved in the trafficking of PrP^C (reviewed in Cashman and Caughey, 2004). We have previously investigated uptake, recycling and processing of the HS-substituted and GPI-anchored proteoglycan (PG) glypican-1 (Gpc-1); for review, see Fransson et al., 2004. This PG, which also localizes to lipid rafts (Watanabe et al., 2004), is internalized via vesicles that contain caveolin-1. During recycling, Gpc-1 undergoes processing which results in extensive removal of the HS chains either enzymatically by heparanase or by non-enzymatic, nitric oxide (NO)-catalyzed deaminative cleavage (Fransson et al., 2004). NO is derived from preformed S-nitroso (SNO) groups in the Gpc-1 protein and can be released by ascorbate (Ding et al., 2002). SNO-formation requires the participation of Cu^{2+} ions. We have also shown that Cu^{2+}–loaded PrP^C can support NO-catalyzed Gpc-1 autodegradation in cell-free experiments and that an interaction between the HS chains of Gpc-1 and PrP^C is essential for copper transfer. Moreover, in prion null fibroblasts, Gpc-1 is devoid of SNO, but SNO-formation can be restored by ectopic expression of PrP^C (Mani et al., 2003). Interestingly, in wild-type mice neuronal NO-synthase is raft-associated, whereas in $PrP^{0/0}$ mice it is not (Keshet et al., 1999).

Here, we have investigated the possibility that interaction between Gpc-1 and Cu^{2+}–loaded PrP^C is required for co-internalization We find that PrP^C is controlling both the internalization of Gpc-1 and its NO-dependent autoprocessing. Silencing of Gpc-1 expression does not affect copper-stimulated PrP^C endocytosis and, conversely, in copper-stimulated cells that express PrP^C lacking the copper-binding domain, internalization of Gpc-1 is much reduced and autoprocessing is abrogated.

EXPERIMENTAL PROCEDURES

Materials

Mouse N2a neuroblastoma cells were obtained from ATCC and maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum. Embryonic fibroblasts from prion null mice ($PRNP^{0/0}$) were a generous gift from Prof. A. Aguzzi, University of Zurich,

Switzerland and wild type mouse embryonic fibroblasts were a generous gift from Dr. R. Cappai, University of Melbourne, Australia. $Na_2{}^{35}SO_4$ (1310 Ci/mmol) was obtained from Amersham International, UK. Dynabeads M-450 rat anti-mouse IgG was obtaind from Dynal ASA, Oslo, Norway. Pwo Polymerase was obtained from Roche, Lewes UK. Monoclonal antibody (mAb) 6H4 against PrP^C was from Prionics, Zurich, Switzerland and antibodies against caveolin-1 was from BD Biosciences, Stockholm, Sweden. The anti-TfR-1 mAb BerT9 was from Dakopatts, Denmark. Immun-Star HRP chemiluminescent kit was purchased from Bio-Rad, USA. Polyclonal antisera against mouse Gpc-1 and mAb recognizing anhydromannose (anMan)-terminating HS oligosaccharides, and suitably tagged secondary antibodies, as well as enzymes, prepacked columns and chemicals were generated or obtained as described previously (Mani et al., 2003, Cheng et al., 2002).

Cell culture and radiolabeling:

N2a cells were cultured as monolayers in MEM supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/mL) and streptomycin (100 µg/mL) in an incubator with humidified atmosphere and 5% CO_2 at 37°C. Confluent cells were incubated low-sulfate, $MgCl_2$-labeling medium supplemented with 2 mM glutamine, 50 mCi/mL of [^{35}S] sulfate for 24 h.

Isolation of caveolin-1 containing compartments using immunomagnetic method

Cells were homogenized in ice cold Triton X-100, 0.25 M sucrose, 0.5 mM phenylmethyl sulfonyl fluoride. BSA was added to a final concentration of 1% (w/v) and caveolae-1 containing compartments were isolated from cell extracts using mouse anti-caveolin-1 (1:1000) as primary antibody and dynabeads M-450 rat anti-mouse IgG (8 x 10^6 beads) as magnetic secondary antibody. The magnetic particles were recovered using a magnetic particle concentrator. The magnetic-isolated material were then solubilized either in 4 M guanidinium choride, 2% TritonX-100 and subjected to gel filtration chromatography on Superose 6 or in SDS-PAGE under reducing conditions followed by Western blotting using the anti-PrP^C mAb (1:2500) for detection.

Isolation of Gpc-1 and gel filtration chromatography

Immunoisolation of Gpc-1 by using anti-Gpc-1 antiserum and protein A-Sepharose was carried out as described previouly (Ding et al., 2001; Cheng et al., 2002). In brief, the samples were extracted with radioimmune precipitiation buffer (phosphate-buffered saline containing 0.1% (w/v) SDS, 0.5% (v/v) Triton X-100, 0.5 % (w/v) sodium deoxycholate) and then treated with protein-A Sepharose CL-4B (1:500) for at least 1 h on a slow shaker at 4°C. The supernatant was collected and treated with polyclonal anti-Gpc-1 antiserum (1:200) at 4°C overnight. Immune complexes were recovered on protein-A Sepharose CL-4B (1:100), which was washed 6 times with 0.15 M NaCl, 10 mM Tris, pH7.4 containing 0.2% (v/v) Tween 20. Bound material were released by 4 M guanidinium choride, 2% TritonX-100 and subjected to gel filtration chromatography on Superose 6. The flow rate was 0.4 ml/min and 0.4 ml fractions were collected. Radioactivity of the collected fractions were analyzed by β-scintillation counting.

Co-immunoprecipitation of Gpc-1 and PrP^C

N2a cells were extracted in 0.1-0.2 ml/Cm^2 dish of 0.15 M NaCl, 10 mM EDTA, 2% (v/v) Triton X-100, 10 mM KH_2PO_4, pH 7.5, 5 µg/ml ovalbumin containing 10 mM N-ethylmaleimide , and 1 mM diisopropylphosphoro-fluoridate on a slow shaker at 4°C for 10 min and then treated with protein-A Sepharose CL-4B (1:500) for at least 1 h on a slow shaker at 4°C. The supernatant was collected and treated with polyclonal anti-Gpc-1 antiserum (1:200) at 4°C overnight. Immune complexes were recovered on protein-A Sepharose CL-4B (1:100), which was washed 6 times with 0.15 M NaCl, 10 mM Tris, pH7.4 containing 0.2% (v/v) Tween 20. Bound material were released by boiling in SDS-buffer and subjected to PAGE under reducing conditions followed by Western blotting using the anti-PrP^C mAb (1:2500).

SDS-PAGE and Western blotting

Samples were subjected to SDS-PAGE on 4-12% gels under reducing conditions and transferred to PVDF membranes as described elsewhere (Mani et al., 2000). After exposure to blocking buffer (PBS

containing 10% non-fat milk, 0.05% Tween 20), membranes were incubated with mAb for PrP^C (1:2,500) or mAb for caveolin-1 (1:2,500) overnight at 4°C. After washing, the membranes were probed with goat anti-mouse horse-radish peroxidase-conjugated antibody (1:10,000) for 1 h at room temperature and developed using a Fujifilm ECL detector.

GFP- PrP^C construct

The open reading frame of the mouse prion protein gene, PrP(m), was cloned into pEGFP-C1 (Clontech – BD Biosciences, Oxford UK) as previously described (Holme et al., 2003). Deletion mutations were introduced into GFP PrP^C using site directed PCR mutagenesis. Mutagenesis to remove the octameric repeat region delta 51-89 was carried out using a PCR based method and two specific splint oligos F 5'GGC AAC CGT TAC CCA GGA GGG GGT ACC CAT3' R 5'CCG TTG GCA ATG GGT CCT CCC CCA TGG GTA 3'. Following a hot start of 10 minutes, 16 PCR cycles were carried out using Pwo Polymerase. Parental DNA was removed by digestion with Dpn1. PCR DNA was then transformed into XL2-Blue Ultra competent cells (Stratagene, USA) and, after purification, sequenced to determine the presence of the mutation.

Trypsin treatment and cell extraction

N2a cells were washed twice with cold PBS and cells were released by trysinization before extraction with Triton X-100. Trypsin digestion was carried out using 20 µg/ml trypsin for 2-3 min at 37 °C. The reaction was terminated by the addition of serum (10%, v/v). The trypsinate/supernatant was recovered by centrifugation at 3000 rpm for 5 min. The cells were then extracted with 0.5 ml of 0.15 M NaCl, 10 mM EDTA, 2% (v/v) Triton X-100, 10 mM KH2PO4, pH 7.5, 5µg/ml ovalbumin containing 10 mM N-ethylmaleimide, and 1 mM diisopopylphosphoro-fluoridate on a slow shaker at 4°C for 10 min. Cell lysates and trypsinates were analyzed separately by slot blot assay.

Slot blot assay

Samples of trypsinates and cell lysates were applied to nitrocellulose membrane in a slot-blot manifold (Schleicher and Schull). The membrane was blocked with 10% non-fat milk at room temperature for 1h then washed and probed with the mAb for PrP^C (1:1000) or polyclonal antisera against mouse Gpc-1 (1:1000) in 3% BSA overnight at 4°C. After washing, the membrane was probed with goat anti-mouse horse-radish peroxidase-conjugated antibody (1:10,000) for PrP^C or with anti-rabbit horse-radish peroxidase-conjugated antibody (1:10,000) for Gpc-1 for 1 h at room temperature and developed with Super Signal for 2 min.

siRNA Preparation

The vector pRNA-U6.1/Neo containing the sequence GTTGGTCTACTGTGCTCAT (corresponding to nucleotides 753-771 in mouse Gpc-1) followed by a hairpin sequence TTCAAGAGA, then the reversed complementary Gpc-1 sequence with an additional C in the 5´-end and a stretch of six T for RNA polymerase III termination followed by GGAA in the 3´-end was synthesized by Genscript Corporation, USA. A negative control vector comprising a scrambled sequence was also prepared.

Transfection method

Transfection was accomplished by using Lipofectamine (Life Technologies) according to the description of the manufacturer.

Confocal laser scanning immunofluorescence microscopy

The various procedures including seeding of cells, fixation, the use of primary and secondary antibodies, generation of images by sequential scans and data processing were the same as those used previously (Ding et al., 2002; Mani et al., 2003; Cheng et al., 2002) or as recommended by the manufacturers. In brief, cells were washed with phosphate buffered saline (PBS) three times and then fixed with acetone for 2-4 min followed permeabilization using 0.5 ml of 2% (v/v) H_2O_2 in 60% (v/v)

methanol for 15 min. After washing with PBS three times for 1 min each, cells were incubated with nonimmune serum (1:100 dilution) or with anti-mouse IgG (1:200) for 30 min at room temperature. Primary antibodies were then applied as described by the manufacturers and kept for 3 h. Cells were then washed three times with PBS and exposed to secondary antibodies (1:500 dilution) for 2 h. The secondary antibodies used were either goat anti-mouse total Ig when the primary antibody was a monoclonal or goat anti-rabbit IgG when the primary antibody was polyclonal. The secondary antibodies were tagged with either fluoresceine isothiocyanate or Texas Red and appropriately combined for colocalization studies. In the controls, the primary antibody was omitted. The images shown were obtained at a focal plane that was at the center of the cell and of 0.3-0.5 µm thickness. Identical exposure settings were used for image capture. Images were digitized and transferred to Adope PhotoShop for merging, annotation and printing.

Flow cytometry

Mouse embryonic fibroblasts and embryonic fibroblasts from prion null mice were seeded in 24-well plates and grown to near confluence in MEM containing glutamine, penicillin, streptomycin and 10% fetal calf serum. Cells were rinsed with medium and detached using trypsin (0.5 ml 0.05% w/v of trypsin in PBS for 1 minute). Trypsinization was terminated by replacing the trypsin solution with 0.5 ml medium supplemented with 10% fetal bovine serum. Cells were recovered by gentle suspension and transferred to tubes, adding 1 vol. of PBS containing 1% BSA (w/v). Cells were then pelleted by centrifugation and resuspended in 0.2 ml PBS after removal of the supernatant. Cells were fixed for 30 minutes in 1 ml PBS containing 4% paraformaldehyde (w/v) for 20 min whilst initially vortexing. Permeabilisation was performed by incubation with 0.2% TritonX-100 in PBS (v/v) for 20 minutes. Immunostaining of the cells with the mAb specific for anMan-containing HS degradation products as the primary antibody and goat antimouse total Ig as the secondary antibody was performed as described for confocal microscopy. In the controls, the primary antibody was omitted. After each step, cells were recovered by centrifugation at 350 x g for 5 minutes. The cells were finally suspended in PBS containing 1% BSA, and analyzed for fluorescence in a fluorescence assisted cell sorting instrument (Calibur, Becton Dickinson Biosciences, USA) operated by Cell-Quest software.

RESULTS

Gpc-1 is localized to caveolin-1-containing vesicles

We have previously demonstrated that NO-dependent processing of HS chains in recycling Gpc-1 takes place in caveolin-1 containing endosomes in T24 cells. Western blot analyses of caveolin-1 in N2a cells demonstrated that these cells have the potential to generate caveolae (Fig. 1A). To determine whether Gpc-1 was localized to caveolae, PG and glycosaminoglycans in N2a cells were metabolically radiolabeled with [^{35}S] sulfate. Caveolin-1 containing compartments were isolated from cold Triton X-100 and 0.25 M sucrose containing cell homogenates by using mouse anti-caveolin-1 as primary antibody and dynabeads M-450 rat anti-mouse IgG as magnetic secondary antibody. When these compartments were solubilized in 4 M guanidinium choride, 2% TritonX-100 and subjected to gel filtration chromatography on Superose 6, two size-pools of radiolabeled material were obtained (Fig. 1B, solid line). As a negative control, precipitation was done with only magnetic secondary antibody. No radioactivity was obtained in the control experiment. In order to determine whether Gpc-1 was present in caveolin containing compartments, the radiolabeled material was pooled, solubilized in phosphate-buffered saline containing 0.1% (w/v) SDS, 0.5% (v/v) Triton X-100, 0.5 % (w/v) sodium deoxycholate and immunoprecipitated using polyclonal anti-Gpc-1 antiserum directed against the Gpc-1 core protein. This material, which may contain Gpc-1 with long HS chains as well as Gpc-1 with truncated chains, was rechromatographed on Superose 6. Almost 80% of the radioactive material was recovered and appeared both as large-size and small-size Gpc-1 (Fig. 1B, dashed line). Previous studies have shown that the long HS side chains in the Gpc-1 precursor can shield epitopes in the core protein and therefore result in lower recovery (Ding et al., 2001).

Figure 1 here

Prion protein colocalizes with Gpc-1 and caveolin-1 containing compartments

As both PrP^C and Gpc-1 are GPI-linked molecules they may colocalize in lipid rafts, caveolae, and caveosomes. In order to determine whether PrP^C colocalizes with caveolin-1 containing

compartments, the N2a cells were transfected with an expression vector for GFP-tagged mouse PrP^C and caveolin-1 containing material were isolated using magnetic immunoisolation. The isolated material were then subjected to SDS-PAGE followed by Western blotting using monoclonal antibodies directed against PrP^C. A negative control without any caveolin-1 magnetic isolation was also included. As shown in Fig. 1C a PrP^C-positive band corresponding to PrP^C appeared at 20 kDa in caveolin-1 immunoisolated material indicating localization of PrP^C to caveolin-1 containing compartments. To further investigate the interaction between Gpc-1 and PrP^C, co-immunopreciptation of Gpc-1 and PrP^C was performed in N2a cell extracts. A negative control without any Gpc-1 immunoisolation was also included. As shown in Fig. 1D, a PrP^C-positive band corresponding to PrP^C appeared at 20 kDa in Gpc-1 immunoisolated material indicating colocalization and interaction between Gpc-1 and PrP^C.

We also used confocal immunofluorescence microscopy to localize PrP^C, Gpc-1 and caveolin-1 in N2a cells. Gpc-1 was detected by a polyclonal antiserum directed against the Gpc-1 core protein and PrP^C either by the mAb 6H4 directed against PrP^C (Fig. 2B and 2C, 6H4) or by transfecting cells with an expression vector for GFP-tagged mouse PrP^C (Fig. 2D and E, GFP-PrP^C). The results showed that Gpc-1 was located close to the cell surface and partially colocalized with caveolin-1 containing vesicles (Fig. 2A, merged panel). By using antibodies directed against PrP^C that detects endogenous intracellular trafficking prion protein and anti-Gpc-1 antiserum, partial colocalization between Gpc-1 and PrP^C was observed (Fig. 2B, merged panel). In the same area there was partial colocalization between PrP^C and caveolin-1 indicating that endogenous PrP^C may be partially localized to caveolin-1 containing vesicles but also located to other endocytotic compartments (Fig. 2C, yellow in merged panel). When the cells were transfected with GFP-tagged PrP^C, an extensive colocalization of Gpc-1 with GFP-tagged PrP^C was detected (Fig. 2D, yellow in merged panels). The GFP-tagged PrP^C was localized to caveolin-1 containing compartments (Fig. 1E). Taken together, these results (Fig. 1 and 2) demonstrate colocalization between Gpc-1 and PrP^C in caveolin-1 rich compartments in untreated N2a cells.

Figure 2 here

The mechanism of endocytosis in N2a cells is still debated. Clathrin- and dynamin-mediated endocytosis has been shown to be one pathway for the internalization of the prion protein, but clathrin independent mechanisms has also been reported to participate in the internalization. We therefore examined colocalization of PrP^C with the non-caveolar clathrin marker, transferrin receptor, using confocal immunofluorescence microscopy. Partial colocalization of GFP-PrP^C with transferrin receptor was detected (data not shown). PrP^C and GFP-PrP^C are thought to follow biosynthetic pathways similar to other GPI-anchored proteins from the ER, Golgi and transport vesicles to the plasma membrane. Subsequent studies have argued that in N2a cells mammalian PrP^C is internalized by one of the non-coated pit mechanisms (Marella et al., 2002; Kaneko et al., 1997). Other studies have shown that PrP^C coexists in the cell surface in raft and non-raft components of the membrane and is rapidly and constitutively endocytosed. At some stage in endocytosis prion protein leaves lipid 'raft' domains to enter non-raft membrane (Sunyach et al., 2003). The mechanisms involved in Cu^{2+}-induced PrP^C endocytosis are poorly understood. Previous studies have shown that mouse N2a neuroblastoma cells contain caveolae like vesicles (Harmey et al., 1995) and Cu^{2+} ions stimulate endocytosis of PrP^C through a caveolin-dependent pathway in these cells (Marella et al., 2002). If clathrin and non-clathrin endocytosis act together is still unclear.

Copper ions induce co-internalization of the prion protein and Gpc-1

We have recently shown that several copper-binding proteins, including the prion protein bind Gpc-1 and modulate the copper/zinc-NO-catalyzed degradation of the HS chains (Mani et al., 2003). The cellular prion protein PrP^C binds Cu^{2+} ions through octapeptide repeat motifs located at its N-terminal region (Brown et al., 1997; Millhauser, 2004), and this interaction induces PrP^C internalization (Pauly and Harris, 1998; Lee et al., 2001).

To investigate the intracellular co-trafficking of PrP^C and Gpc-1, N2a cells were transfected with a GFP-tagged version of PrP^C and a GFP-tagged N-terminal deletion mutant (Δ 51-89) of PrP^C lacking the octameric copper-binding repeat and were then treated with 100 µM $CuCl_2$ for 5 to 30 min. The amount of trypsin released cell surface PrP^C and Gpc-1 and detergent soluble intracellular PrP^C and Gpc-1 were detected using antibodies directed against the respective protein in a slot blot assay. In

untreated cells both PrP^C and Gpc-1 were accessible to trypsin (Fig 3A). The amount of trypsin-accessible PrP^C and Gpc-1 decreased after 5-10 min of copper treatment. When the cells were treated with 100 µM $CuCl_2$ for 30 min almost no PrP^C was accessible to trypsin while some Gpc-1 was still released. Copper treatment for 5-10 min increased the amount of PrP^C and Gpc-1 in the cell extracts. Treatment with 100 µM $CuCl_2$ for 30 min induced transition of both PrP^C and Gpc-1 from the cell surface to the cell interior indicating that copper treatment induces internalization of both PrP^C and Gpc-1. GFP-PrP^C devoid of the copper binding N-terminal domain did not shift to the detergent soluble cell lysate after incubation with copper ions for 30 min indicating that binding of copper to the octarepeat region of PrP^C is important for internalization of PrP^C (data not shown).

The cellular localization after copper induced internalization of Gpc-1 and prion protein was analyzed in N2a cells transfected with GFP-tagged version of PrP^C by using confocal immunofluorescence microscopy. Immunolocalization of Gpc-1 and GFP-tagged PrP^C showed colocalization at the cell surface and in vesicles located close to the cell surface (Fig. 3B). Treatment with 100 µM $CuCl_2$ for 5 min induced endocytosis of both Gpc-1 and PrP^C to vesicles located near the cell surface in some cells (Fig. 3C). When the cells were treated with 100 µM $CuCl_2$ for 10 min more extensive internalization of both Gpc-1 and PrP^C with colocalization in vesicles located to para-nuclear regions was observed (Fig. 3D). Treatment with 100 µM $CuCl_2$ for 30 min induced co-internalization of both Gpc-1 and PrP^C with extensive colocalization in the perinuclear compartments (Fig. 3E). Treatment with 50 µM $CuCl_2$ for 30 minutes also induced endocytosis of both Gpc-1 and PrP^C with colocalization in vesicles located to para- and perinuclear regions (data not shown).

Figure 3 here

To further investigate the interaction between Gpc-1 and PrP^C in copper treated cells co-immunopreciptation of Gpc-1 and PrP^C was performed in N2a cell extracts transfected with GFP-PrP^C and then left untreated or treated with 100 µM $CuCl_2$ for 10 and 30 min. A negative control without any Gpc-1 immunoisolation was also included. As shown in Fig. 3F, in untreated cells a PrP^C-positive band corresponding to PrP^C appeared at 20 kDa in Gpc-1 immunoisolated material in untreated cells

indicating interaction between Gpc-1 and PrP^C. The PrP^C-positive band was also detected when the cells were treated with $CuCl_2$ for 10 and 30 min supporting the idea that Gpc-1 and PrP^C co-internalize upon copper treatment.

Syndecans are another family of cell surface HSPG involved in cellular signaling (Tkachenko et al., 2005). It has been demonstrated that plasma membrane syndecans recycle through endosomal compartments (Zimmermann et al., 2005). We therefore tested whether copper treatment induces co-internalization with PrP^C. In untreated cells, syndecan-1 colocalized with GFP-PrP^C at the cell surface to some extent (results not shown). Treatment with 100 µM $CuCl_2$ induced internalization of PrP^C whereas syndecan-1 remained at the cell surface (Fig. 3G) indicating that copper ions do not induce co-internalization of PrP^C and syndecan-1.

Based on the assumption that copper mediates its effect by binding to the octarepeat region of PrP^C, we analyzed the effect of $CuCl_2$ on cells transfected with GFP-tagged N-terminal deletion mutant (Δ 51-89) of PrP^C lacking the octameric copper-binding repeat using confocal immunofluorescence microscopy. Gpc-1 and PrP^C devoid of the N-terminal domain colocalized on the cell surface (Fig. 4A). No change in the distribution pattern of either Gpc-1 or PrP^C was observed when cells transfected with the GFP-tagged N-terminal deletion mutant (Δ 51-89) of PrP^C were treated with 100 µM $CuCl_2$ suggesting that Cu^{2+} loaded PrP^C is important for co-internalization (Fig. 4A and B).

Figure 4 here

In order to investigate whether the internalization effect is specific for Cu^{2+} ions we treated the GFP-PrP^C transfected cells with $NiCl_2$ or $MgCl_2$ at a concentration of 100 µM. Both Gpc-1 and GFP-PrP^C remained at the cell surface and in vesicles located close to the cell surface upon $NiCl_2$ or $MgCl_2$ treatment (Fig. 4C and D) supporting the idea that Cu^{2+} loaded PrP^C is important for internalization. We also studied the endocytic targeting of GFP-PrP^C and Gpc-1 in Cu^{2+} stimulated cells using the endosomal marker Rab 7. Partial colocalization between GFP-PrP^C and Rab 7 was observed in the perinuclear compartments indicating that they were partially located to late endosomes (Fig. 4E).

To evaluate the possibility that Gpc-1 affects PrP^C internalization, expression of Gpc-1 was silenced using siRNA technology. Western blot analysis demonstrated down regulation of Gpc-1 expression to 30%-35% of the normal levels 48 hours after transfection (inserts in Fig. 5A and 5C). When Gpc-1 expression was silenced, PrP^C was still observed in vesicles located close to the cell surface (Fig. 5C, 6H4). Since the expression of Gpc-1 was silenced a very faint colocalization signal was detected in merged panel. Treatment with 50 µM $CuCl_2$ for 30 minutes induced internalization of PrP^C in both mock and siRNA-transfected cells (Fig. 5B and D) indicating that internalization of PrP^C is not dependent on Gpc-1 expression.

Figure 5 here

PrP^C involvement in S-nitroso-dependent autocleavage of Gpc-1 HS

We have previously shown that Cu^{2+} ions are required to nitrosylate thiol groups in the core protein of Gpc-1. When S-nitrosylated Gpc-1 is then exposed to an appropriate reducing agent, such as ascorbate, NO is released and autocatalyzes deaminative cleavage of the Gpc-1 HS side-chains at sites where the glucosamines are N-unsubstituted. This results in conversion of the glucosamines to reducing terminal anhydromannose (anMan) in the liberated HS oligosaccharide fragments. These processes take place in a step-wise manner while Gpc-1 recycles via a caveolin-1-associated pathway where Cu^{2+}-loaded PrP^C can deliver copper-ions to Gpc-1 to support S-nitrosylation (Mani et al., 2003).

To demonstrate that PrP^C supports NO-catalyzed cleavage of HS we examined the formation anMan-containing HS degradation products in wild type mouse embryonic fibroblasts and in embryonic fibroblasts derived from mice with a targeted disruption of the PrP^C gene ($PrP^{0/0}$). Subconfluent wild type mouse embryonic fibroblasts generated anMan-containing HS oligosaccharides (Fig. 6A and B). Gpc-1 was located in vesicles near the cell surface but also inside the cytoplasm and in para- and peri-nuclear compartments. The oligosaccharides were scattered in the cytoplasm and in paranuclear compartments co-localizing with Gpc-1 (Fig. 6C). Prion null fibroblasts expressed Gpc-1 intracellularly but there were almost no anMan-containing HS degradation products (Fig. 6D-F). The difference in anMan production between wild type and prion null cells was significant as determined by flow cytometry. In order to sustain NO-dependent depolymerization of

HS, prion null cells were exposed to sodium nitroprusside (SNP) as NO donor and ascorbate to release NO both in the absence or presence of Cu^{2+} ions. As shown in Fig. 6G-I, there was no production of anMan-containing HS degradation products when the cells were exposed to SNP and ascorbate. However addition of $CuCl_2$ resulted in significant (Fig. 6J, as determined by flow cytometry) and extensive depolymerization of HS with formation of anMan containing products (Fig. 6J-K). The oligosaccharides formed after this treatment were mostly located perinuclearly and intranuclearly and co-localized with Gpc-1 in these locations (Fig. 6L). These results indicate that Cu^{2+} ions are required for NO-dependent degradation of HS chains.

Figure 6 here

To demonstrate that the copper binding property of PrP^C is involved in the degradation of HS chains, the prion null fibroblasts were transfected with GFP tagged PrP^C or with the GFP tagged N-terminal deletion mutant (Δ51-89) of PrP^C that lacks the octameric copper-binding repeat. GFP-PrP^C (Fig. 7A) was predominantly located to vesicles inside the cytoplasm in these cells probably because the trace of $CuSO_4$ in the culture medium is enough for internalization in these cells. When prion null fibroblasts were transfected with GFP-PrP^C there was significant formation of anMan-containing degradation products (Fig. 7A-B) whereas in cells transfected with the N-terminal deletion mutant, no anMan containing products could be detected (Fig. 7C-D). These results indicate that PrP^C can serve as a copper supplier and thereby support NO-catalyzed deaminative cleavage of HS in Gpc-1.

Figure 7 here

DISCUSSION

PrP^C is a GPI-anchored protein that binds Cu^{2+} and HS/heparin. Cu^{2+} binding to PrP^C stimulates endocytosis and recycling of PrP^C (Pauly and Harris, 1998; Martins et al., 2002). It has been debated that PrP^C can be converted to the abnormal, aggregation-prone scrapie isoform (PrP^{Sc}) during internalization via caveolae-dependent pathway (Prusiner, 1998). Three regions of PrP^C, encompassing residues 23-52, 53-91, and 110-128, are able to bind heparin and HS (Warner et al., 2002). HS chains

have been identified as critical factors in amyloidogenesis (McBride et al., 1998; Elimova et al., 2004). The effect of HS seems to be paradoxical as they can both stimulate and inhibit PrP^{Sc} formation. In scrapie infected cell cultures and in animal models, exogenous HS is a strong inhibitor of PrP^{Sc} accumulation (Priola and Caughey, 1994; Schonberger et al., 2003).

The present results show that GPI-anchored PrP^{C} colocalizes with the similarly GPI-linked and HS-substituted Gpc-1 in caveolin-1 enriched compartments. We have previously demonstrated that prion protein provides copper ions required to nitrosylate thiol groups in the core protein of Gpc-1 (Mani et al., 2003). Here we demonstrate co-internalization of PrP^{C} and Gpc-1 from the cell surface to perinuclear compartments induced by exposure to Cu^{2+} ions. When prion null cells are transfected with a PrP^{C} N-terminal deletion mutant lacking the copper-binding sites, both PrP^{C} and Gpc-1 are found at the cell surface and internalization is not induced by Cu^{2+} ions. The available data suggest the possibility that interaction between Gpc-1 and Cu^{2+}-loaded PrP^{C} is required for co-internalization via a caveolar route. Previous studies have shown that in mouse N2a neuroblastoma cells, Cu^{2+} ions stimulate endocytosis of PrP^{C} through a caveolin-dependent pathway (Marella et al., 2002). Other studies have shown that in primary cultured neurons and in mouse N2a neural cells, raft-associated PrP^{C} leaves lipid raft domains to enter non-raft membrane, from which it enters coated pits (Sunyach et al., 2003). Still the mechanism of endocytosis in N2a cells remains to be further investigated.

We demonstrate that silencing of Gpc-1 expression has no effect on copper-stimulated PrP^{C} endocytosis and, conversely, in copper-stimulated cells expressing PrP^{C} lacking the copper binding domain, internalization of Gpc-1 is much reduced suggesting that PrP^{C} is controlling the internalization of Gpc-1. We also find that HS-chains of Gpc-1 are poorly degraded in prion null fibroblasts. Addition of copper ions, NO-donor and ascorbate or ectopic expression of PrP^{C} restores HS degradation in these cells. These results show that PrP^{C} is an important supplier of Cu^{2+} ions and can thereby support NO-catalyzed degradation of HS-chains in recycling Gpc-1.

On the basis of the results presented, we propose a working model for the functional interplay between Gpc-1 and PrP^{C}. Gpc-1 with three HS chains colocalizes with Cu^{2+}-loaded PrP^{C} at the cell surface. Interaction between PrP^{C} and the HS side chains results in transfer of Cu^{2+} from PrP^{C} to Gpc-1 and induces co-internalization. NO synthase that is present at the cytosolic surface of the membrane

generates NO from Arg, and Gpc-1 becomes S-nitrosylated, whereas Cu^{2+} is reduced to Cu^{+}. The S-nitroso groups of Gpc-1 appear to remain stable until Gpc-1 is exposed to a suitable reducing environment where NO is released and anMan-containing HS-oligosaccharides are generated by deaminative cleavage. The triggering mechanisms for NO-release are still unknown. It is intriguing that late endosomes exhibit significant reducing activity (Fivaz et al., 2002), which could support NO release from SNO groups.

The complex formed between PrP^{C}, copper and Gpc-1 HS may have a pivotal role in prion protein movement between cells. It has been shown that HS chains are an essential part of the cellular receptor used for prion uptake (Schonberger et al., 2003; Hijazi et al., 2005; Horonchik et al., 2005). Recent studies have shown that glypicans are involved in passing wingless signal on to neighboring cells, either for paracrine signaling or for further transport (Franch-Marro et al., 2005). Binding of prion proteins to the HS chains of recycling Gpc-1 could contribute to the observed transfer of prion protein by cell to cell contact.

Acknowledgements

We thank Prof. Lars-Åke Fransson at the Department of Experimental Medical Science, Lund University for advice. We also thank Prof. Catharina Svanborg at the Department of Microbiology, Immunology and Glycobiology, Lund University, for access to microscope facilities and Dr Gunnar Peijler, Uppsala University, Sweden for supply of monoclonal antibodies. The work was supported by grants from the Swedish Science Council (VR-M), the Swedish Cancer Fund, the Association for International Cancer Research, the American Cancer Society, the Segerfalk, Bergvall, Crafoord, Hedborg, Kock, Zoega and Österlund Foundations, and the Medical Faculty of Lund University.

FIGURE LEGENDS

FIG. 1. Gpc-1 and PrP^C in caveolin-1-containing vesicles. (A) Western blot analysis of caveolin-1 in N2a cells. (B, solid line) Superose 6 chromatography of [^{35}S] sulfate labeled PG and glycosaminoglycans immunoisolated from cold Triton X-100- and 0.25 M sucrose-containing N2a cell extracts, using mouse anti-caveolin-1 as primary antibody and dynabead M-450 rat anti-mouse IgG as magnetic secondary antibody. (B, dashed line) Radioactively labeled immunoisolated material were pooled as indicated, immunoprecipitated using polyclonal anti-Gpc-1 antiserum and rechromatographed on Superose 6. (C) Co-immunoisolation of caveolin-1 and PrP^C in GFP-PrP^C transfected N2a cell extracts was carried out without (-) or with (+) monoclonal anti-caveolin-1 antibodies followed by magnetic isolation, SDS-PAGE and Western blotting using the anti-PrP^C mAb (6H4). (D) Co-immunopreciptation of Gpc-1 and PrP^C in N2a cell extracts was carried out without (-) or with (+) anti-Gpc-1 antiserum followed by absorption to protein A-Sepharose, SDS-PAGE and Western blotting using the anti- PrP^C mAb (6H4). The amount of protein in the cell extract used for immunoisolation was 0.8 mg. All isolated material was loaded on SDS-PAGE.

FIG. 2. Prion protein colocalizes with Gpc-1 in caveolin-1 containing compartments in N2a cells. The panels show confocal laser immunofluorescence microscopy of (A) Gpc-1 (red) and caveolin 1 (green), (B) Gpc-1 (red) and PrP^C (green, 6H4), and (C) caveolin-1 (red) and PrP^C (green, 6H4) in N2a cells. (D and E) The same cells were transiently transfected with a plasmid containing cDNA for mouse PrP^C fused to green fluorescent protein (GFP- PrP^C) for 48 h. Confocal laser immunofluorescence microscopy of (D) Gpc-1 (red) and GFP- PrP^C (green) show colocalization at the cell surface and in vesicles located close to the cell surface. Confocal laser immunofluorescence microscopy of (C) caveolin 1 (red) and PrP^C (6H4) (green) or (E) caveolin 1 (red) and GFP- PrP^C (green) also show similar colocalization. Bar, 20 μm. In areas (square) of higher magnification (100x) colocalizations are shown.

FIG. 3. Copper ions induce co-internalization of prion protein and Gpc-1. The panels show N2a cells transiently transfected with GFP-PrP^C for 48 hours. (A) Slot blot assay of trypsin-released cell surface PrP^C and Gpc-1 and detergent soluble intracellular PrP^C and Gpc-1 in cells transfected with GFP-PrP^C in the absence or presence of 100 µM $CuCl_2$ for different time periods. GFP-PrP^C transfected N2a cells were (B) left untreated or were treated with 100 µM $CuCl_2$ for (C) 5 min, (D) 10 min, and (E) 30 minutes at 37 °C. Confocal laser immunofluorescence microscopy of (B) Gpc-1 (red) and GFP- PrP^C (green) show colocalization at the cell surface and in vesicles located close to cell surface. (E) Treatment with $CuCl_2$ induces endocytosis of Gpc-1 and PrP^C with strong colocalization in the para- and perinuclear regions. (F) Co-immunopreciptation of Gpc-1 and PrP^C in N2a cell extracts transfected with GFP-PrP^C and then left untreated or treated with 100 µM $CuCl_2$ for 10 and 30 min. Immunoisolation was carried out without (-) or with (+) anti-Gpc-1 antiserum followed by absorption to protein A-Sepharose, SDS-PAGE and Western blotting using the anti- PrP^C mAb (6H4). The amount of protein in the cell extract used for immunoisolation was 0.8 mg. All isolated material was loaded on SDS-PAGE. (E) No colocalization between syndecan-1 (red) and GFP- PrP^C (green) was detected after $CuCl_2$ treatment. Bar, 20 µm.

FIG 4. Role of copper loaded prion protein on internalization. (A-B)The panels show N2a cells transiently transfected with GFP-PrP^C Δ 51-89 for 48 hours. (A) Cells were then left untreated, or (B) were treated with 100 µM $CuCl_2$ for 30 minutes at 37 °C. Confocal laser immunofluorescence microscopy of (A) Gpc-1 (red), GFP- PrP^C Δ51-89 (green) show colocalization in vesicles located close to the cell surface. (B) No change in the distribution pattern of either Gpc-1 or PrP^C was observed when cells transfected with GFP- PrP^C Δ 51-89 were treated with $CuCl_2$. Bar, 20 µm. (C-E) The panels show N2a cells transiently transfected with GFP- PrP^C for 48 hours. Cells were treated with (C) 100 µM $NiCl_2$, (D) 100 µM $MgCl_2$, or (E) 50 µM $CuCl_2$ for 30 minutes at 37 °C. Confocal laser immunofluorescence microscopy of Gpc-1 (red), GFP-PrP^C (green) show colocalization in vesicles close to the cell surface in cells treated with $NiCl_2$ or $MgCl_2$. Confocal laser

immunofluorescence microscopy of (E) Rab-7 (red) and GFP-PrP^C (green) show colocalization in vesicles located close to nucleus in $CuCl_2$ treated cells. Bar, 20 µm.

FIG. 5. Copper induced internalisation of PrP^C in N2a cells when Gpc-1 expression is silenced. The panels show confocal laser immunofluorescence microscopy of Gpc-1 and PrP^C (6H4) in N2a cells. The cells were transiently transfected with (A and B) unspecific or (C and D) Gpc-1-specific siRNA and left (A and C) untreated for 72 h or (B and D) were treated with 50 µM $CuCl_2$ during the last 30 minutes. Bar, 20 µM. Inserts in A and C demonstrate western blot analysis of Gpc-1 in N2a cells transiently transfected with (A) unspecific or (C) Gpc-1-specific siRNA. 35 µg of total protein was loaded onto the gel in each case and Western blotting was performed with anti-Gpc-1 antiserum.

FIG. 6. Quantification and immunolocalization of Gpc-1 and anMan-containing HS degradation products in mouse embryonic fibroblasts from (A-C) wild type or (D-L) prion knock out mice. Cells were either left (A-F) untreated or were treated with (G-I) 1 mM SNP and 1 mM ascorbate, or (J-L) 1 mM SNP, 1 mM ascorbate and 100 µM $CuCl_2$ for 1 h. Results of flow cytometry measurements after staining for anMan-containing HS oligosaccharides are shown in A-D-G-J and expressed as fluorescence intensity. The error bars indicate standard error of the mean. ***, $p<0.001$. The confocal laser immunofluorescence microscopy images (B, E, H, K) at low magnification (10x) were obtained after staining for anMan-containing oligosaccharides. (C, F, I, L) In areas (square) of higher magnification (100x) colocalizations between Gpc-1 (red) and anMan-containing oligosaccharides (green) are shown. Bar 20 µm.

FIG. 7. PrP^C involvement in the autocleavage of Gpc-1 HS. Confocal laser immunofluorescence microscopy of prion null mouse embryonic fibroblasts transiently transfected with (A and B) GFP- PrP^C or with (C and D) GFP-PrP^C Δ 51-89 for 48 hours. The panels show confocal laser immunofluorescence microscopy for (A and C, green) PrP^C and (B and D, red) anMan-containing oligosaccharides. Bar, 20 µM.

REFERENCES

Aguzzi A. and Polymenidou M. (2004) Mammalian prion biology: one century of evolving concepts. *Cell* **116**, 313-327.

Brown D. R., Qin K., Herms J. W., Madlung A., Manson J., Strome R., Fraser P. E., Kruck T., von Bohlen A., Schulz-Scheffer W., Giese A., Westaway D., and Kretzschmar H. (1997) The cellular prion protein binds copper in vivo. *Nature* **390**, 684-687.

Brown D. R. (1999) Prion protein expression aids cellular uptake and veratridine-induced release of copper. *J. Neurosci. Res.* **58**, 717-725.

Brown D. R., Wong B. S., Hafiz F., Clive C., Haswell S., and Jones I. M. (1999) Normal prion protein has an activity like that of superoxide dismutase. *Biochem. J.* **344**, 1-5.

Brown D. R. (2001). Prion and prejudice: normal protein at the synapse. *Trends Neurosci.* **24,** 85-90.

Brown L. R. and Harris D. A. (2003). Copper and zinc cause delivery of the prion protein from the plasma membrane to a subset of early endosomes and the Golgi. *J. Neurochem.* **87**, 353-363.

Cashman N. R. and Caughey B. (2004) Prion diseases--close to effective therapy? *Nature Rev. Drug Discovery* **3**, 874-884.

Cheng F., Mani K., van den Born J., Ding K., Belting M., and Fransson L. Å. (2002). Nitric oxide-dependent processing of heparan sulfate in recycling S-nitrosylated glypican-1 takes place in caveolin-1-containing endosomes. *J. Biol. Chem.* **277**, 44431-44439.

Ding K., Sandgren S., Mani K., Belting M., and Fransson L. Å. (2001) Modulations of glypican-1 heparan sulfate structure by inhibition of endogenous polyamine synthesis. Mapping of spermine-binding sites and heparanase, heparin lyase, and nitric oxide/nitrite cleavage sites. *J. Biol. Chem.* **276**, 46779-46791.

Ding K., Mani K., Cheng F., Belting M., and Fransson L. Å. (2002) Copper-dependent autocleavage of glypican-1 heparan sulfate by nitric oxide derived from intrinsic

nitrosothiols. *J. Biol. Chem.* **277,** 33353-33360.

Elimova E., Kisilevsky R., Szarek W. A., and Ancsin J. B. (2004) Amyloidogenesis recapitulated in cell culture: a peptide inhibitor provides direct evidence for the role of heparan sulfate and suggests a new treatment strategy. *FASEB J.* **14**, 1749-1751.

Fivaz M., Vilbois F., Thurnheer S., Pasquali C., Abrami L, Bickel P. E., Parton R. G., van der Goot F. G. (2002) Differential sorting and fate of endocytosed GPI-anchored proteins. *EMBO J.* **15**,3989-4000.

Franch-Marro X., Marchand O., Piddini E., Ricardo S., Alexandre C., and Vincent J. P. (2005) Glypicans shunt the Wingless signal between local signalling and further transport. *Development* **132,** 659-666.

Fransson L. Å., Belting M., Cheng F., Jönsson M., Mani K., and Sandgren S. (2004) Novel aspects of glypican glycobiology. *CMLS Cell Mol. Life Sci.* **61**, 1016-1024.

Harmey J. H., Doyle D., Brown V., Rogers M. S. (1995) The cellular isoform of the prion protein, PrPc, is associated with caveolae in mouse neuroblastoma (N2a) cells.*Biochem Biophys Res Commun.* **210**, 753-759.

Hijazi, N., Kariv-Inbal, Z., Gasset, M., Gabizon, R. (2005) PrPSc incorporation to cells requires endogenous glycosaminoglycan expression. *J. Biol. Chem.* 17, 17057-17061.

Holme A., Daniels M., Sassoon J., and Brown D. R. (2003) A novel method of generating neuronal Holme cell lines from gene-knockout mice to study prion protein membrane orientation. *Eur. J. Neurosci.* **18**, 571-579.

Horonchik L., Tzaban S., Ben-Zaken O., Yedidia Y., Rouvinski A., Papy-Garcia D., Barritault D., Vlodavsky I., and Taraboulos A. (2005) Heparan sulfate is a cellular receptor for purified infectious prions. *J. Biol .Chem.* **280,** 17062-17067.

Kaneko K., Vey M., Scott M., Pilkuhn S., Cohen F. E. and Prusiner S. B. (1997) COOH-terminalsequence of the cellular prion protein directs subcellular trafficking and controls conversion into the scrapie isoform. *Proc. Natl. Acad. Sci. USA,* **94**, 2333-2338.

Keshet G. I., Ovadia H., Taraboulos A., and Gabizon R. (1999) Scrapie-infected mice and PrP knockout mice share abnormal localization and activity of neuronal nitric oxide

synthase.*J. Neurochem.* **72**, 1224-1231.

Lee K. S., Magalhaes A. C., Zanata S. M., Brentani R. R., Martins V. R., and Prado M. A. M. (2001) Internalization of mammalian fluorescent cellular prion protein and N-terminal deletion mutants in living cells. *J. Neurochem.* **79,** 79-87.

Mange A., Milhavet O., Umlauf D., Harris D., and Lehmann S. (2002) PrP-dependent cell adhesion in N2a neuroblastoma cells. *FEBS Lett.* **514**, 159-162.

Mani K., Jonsson M., Edgren G., Belting M., and Fransson L. Å. (2000) A novel role for nitric oxide in the endogenous degradation of heparan sulfate during recycling of glypican-1 in vascular endothelial cells. *Glycobiology* **6**, 577-586.

Mani K., Cheng F., Havsmark B., Jönsson M., Belting M., and Fransson L. Å. (2003) Prion, amyloid beta-derived Cu(II) ions, or free Zn(II) ions support S-nitroso-dependent autocleavage of glypican-1 heparan sulfate. *J. Biol. Chem.* **278**, 38956-38965.

Marella M., Lehmann S., Grassi J., and Chabry J. (2002) Filipin prevents pathological prion protein accumulation by reducing endocytosis and inducing cellular PrP release. *J. Biol. Chem.* **277**, 25457-25464.

Martins V. R., Linden R., Pardo M. A. M., Walz R., Sakamoto A. C., Izquierdo I., and Brentani R. R. (2002). Cellular prion protein: on the road for functions. *FEBS Lett.* **512**, 25-28.

McBride P. A., Wilson M. I., Eikelenboom P., Tunstall A., and Bruce M. E. (1998) Heparan sulfate proteoglycan is associated with amyloid plaques and neuroanatomically targeted PrP pathology throughout the incubation period of scrapie-infected mice. *Exp. Neurol.* **149,**447-454.

Millhauser G. L. (2004). Copper binding in the prion protein. *Acc. Chem. Res,* 2, 79-85.

Mouillet-Richard S., Ermonval M., Chebassier C., Laplanche J.L., Lehmann S., Launay J. M., and Kellermann O. (2000) Signal transduction through prion protein. *Science* **289**, 1925-1928.

Nichols B. (2003) Caveosomes and endocytosis of lipid rafts. *J. Cell. Sci.* **116**, 4707-4714.

Pauly P. C. and Harris D. A. (1998) Copper stimulates endocytosis of the prion protein. *J. Biol. Chem.* **273**, 33107-33110.

Pelkmans L. and Helenius A. (2002) Endocytosis via caveolae. *Traffic* **3**, 311-320.

Peters P. J., Mironov A., Peretz D., van Donselaar E., Leclerc E., Erpel S., DeArmond S. J., Burton D. R., Williamson R. A., Vey M., and Prusiner S. B. (2003) Trafficking of prion proteins through a caveolae-mediated endosomal pathway. *J. Cell. Biol.* **162**, 703-717.

Priola S. A. and Caughey B. (1994) Inhibition of scrapie-associated PrP accumulation. Probing the role of glycosaminoglycans in amyloidogenesis. *Mol. Neurobiol.* **8**, 113-120.

Prusiner S. B. (1998) Prions. *Proc. Natl. Acad. Sci. USA* **95**, 13363-13383.

Qin K., Yang Y., Mastrangelo P., and Westaway D. (2002) Mapping Cu(II) binding sites in prion proteins by diethyl pyrocarbonate modification and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometric footprinting. *J. Biol. Chem.* **277**, 1981-1990.

Schonberger O., Horonchik L., Gabizon R., Papy-Garcia D., Barritault D., and Taraboulos A. (2003) Novel heparan mimetics potently inhibit the scrapie prion protein and its endocytosis. *Biochem. Biophys. Res. Commun.* **312**, 473-479.

Shyng S. L., Moulder K. L., Lesko A., and Harris D. A. (1995) The N-terminal domain of a glycolipid-anchored prion protein is essential for its endocytosis via clathrin-coated pits. *J. Biol . Chem.* **270,** 14793-14800.

Sunyach C., Jen A., Deng J., Fitzgerald K. T., Frobert Y., Grassi J., McCaffrey M. W., Morris R. (2003) The mechanism of internalization of glycosylphosphatidylinositol-anchored prion protein. *EMBO J.* **22**, 3591-3601.

Tkachenko E., Rhodes J. M., and Simons M. (2005) Syndecans: new kids on the signaling block. *Circ. Res.,* **96**, 488-500.

Vey M., Pilkuhn S., Wille H., Nixon R., DeArmond S., Smart E. J., Anderson R. G. W., Taraboulos A., and Prusiner S. B. (1996) Subcellular colocalization of the cellular and scrapie prion proteins in caveolae-like membranous domains. *Proc. Natl. Acad. Sci. USA* **93**,14945-14949.

Viles J. H., Cohen F. E., Prusiner S. B., Goodin D. B., Wright P. E., and Dyson H. J. (1999) Copper binding to the prion protein: structural implications of four identical cooperative binding sites. *Proc. Natl. Acad. Sci. USA* **96**, 2042-2047.

Warner R. G., Hundt C., Weiss S., and Turnbull J. E. (2002) Identification of the heparan sulfate binding sites in the cellular prion protein. *J. Biol. Chem.* **277**, 18421-18430.

Watanabe N., Araki W., Chui D. H., Makifuchi T., Ihara Y., and Tabira. T. (2004) Glypican-1 as an Abeta binding HSPG in the human brain: its localization in DIG domains and possible roles in the pathogenesis of Alzheimer's disease. *FASEB ,* **18**,1013-1015.

Zimmermann P., Zhang Z., Degeest G., Mortier E., Leenaerts I., Coomans C., Schulz J., N'Kuli F., Courtoy P.J., David G. (2005) Syndecan recycling [corrected] is controlled by syntenin-PIP2 interaction and Arf6. *Dev. Cell. ,* **3**, 377-88.

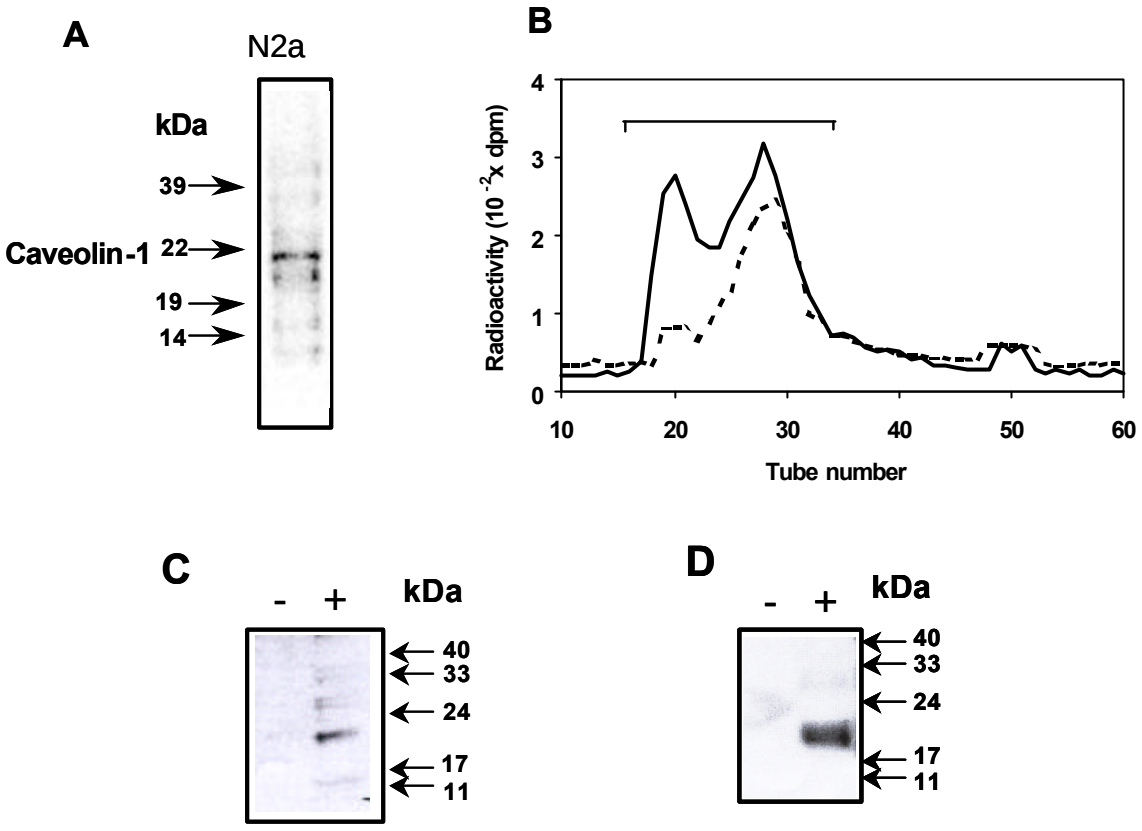
A
N2a
kDa
39
Caveolin-1 22
19
14
B
Radioactivity (10 -2 x dpm)
4
3
2
1
0
10
20
30
40
50
60
Tube number
C
- +
kDa
40
33
24
17
11
D
- +
kDa
40
33
24
17
11

Fig. 1

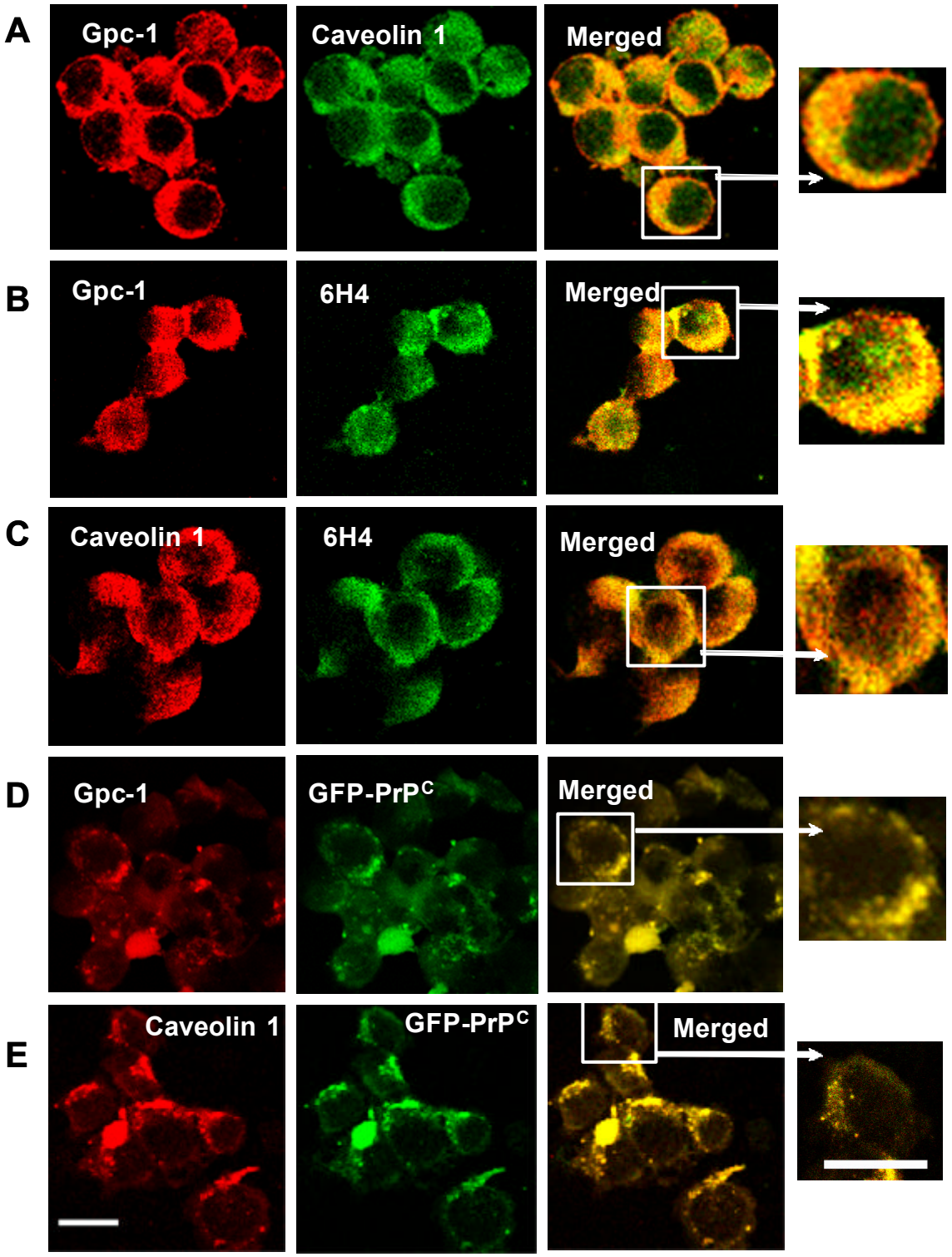
A
Gpc-1
Caveolin 1
Merged
B
Gpc-1
6H4
Merged
C
Caveolin 1
6H4
Merged
D
Gpc-1
GFP-PrP^{C}
Merged
E
Caveolin 1
GFP-PrP^{C}
Merged

Fig. 2

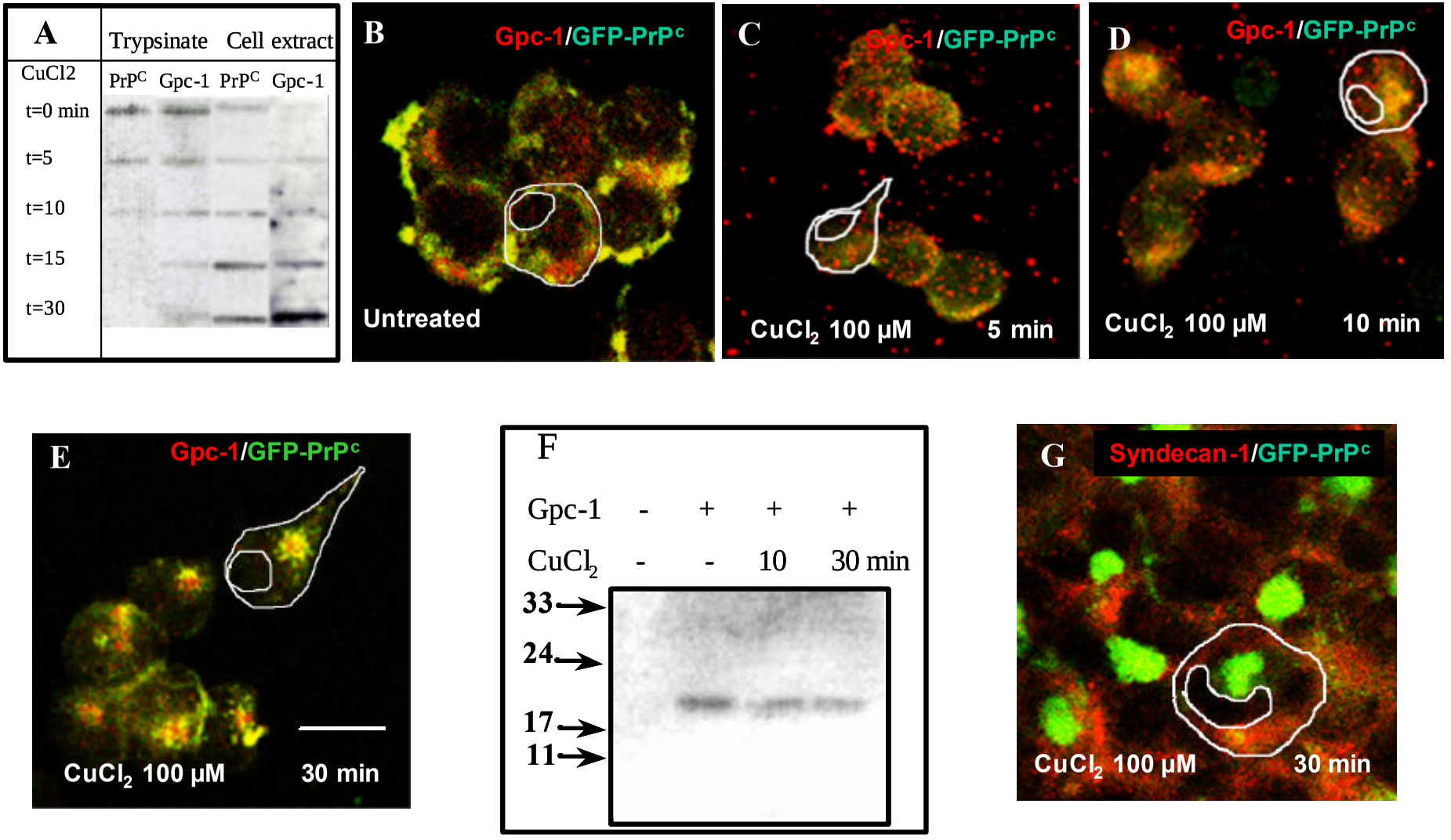
A
Trypsinate
Cell extract
CuCl2
PrP^C
Gpc-1
PrP^C
Gpc-1
t=0 min
t=5
t=10
t=15
t=30
B
Gpc-1/GFP-PrP^c
Untreated
C
Gpc-1/GFP-PrP^c
CuCl2 100 µM
5 min
D
Gpc-1/GFP-PrP^c
CuCl2 100 µM
10 min
E
Gpc-1/GFP-PrP^c
CuCl2 100 µM
30 min
F
Gpc-1 - + + +
CuCl2 - - 10 30 min
33
24
17
11
G
Syndecan-1/GFP-PrP^c
CuCl2 100 µM
30 min

Fig. 3

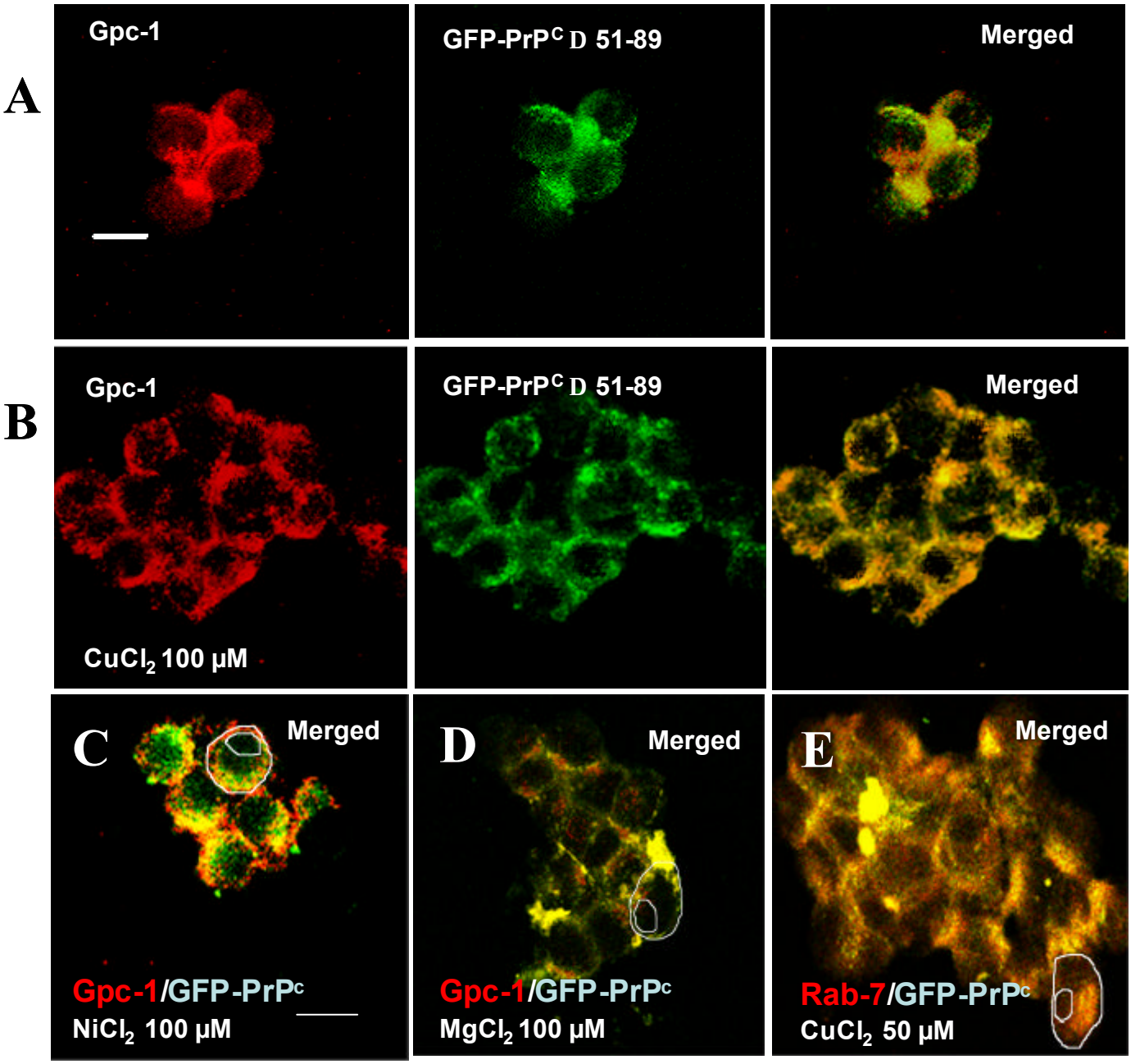
A
Gpc-1
GFP-PrP^C D 51-89
Merged
B
Gpc-1
GFP-PrP^C D 51-89
Merged
CuCl2 100 µM
C
Merged
Gpc-1/GFP-PrPc
NiCl2 100 µM
D
Merged
Gpc-1/GFP-PrPc
MgCl2 100 µM
E
Merged
Rab-7/GFP-PrPc
CuCl2 50 µM

Fig. 4

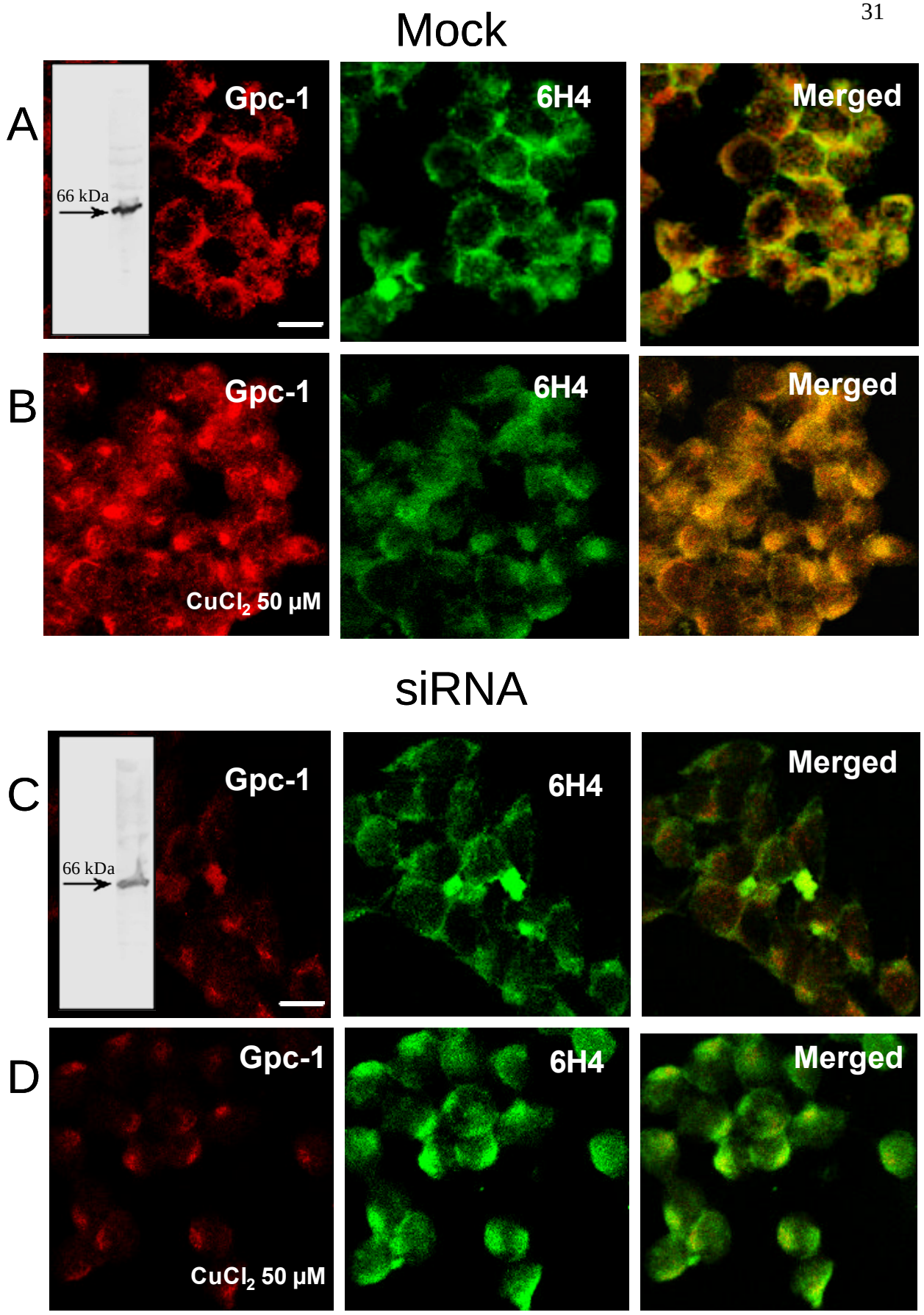
Mock
A
Gpc-1
66 kDa
6H4
Merged
B
Gpc-1
CuCl2 50 μM
6H4
Merged
siRNA
C
Gpc-1
66 kDa
6H4
Merged
D
Gpc-1
CuCl2 50 μM
6H4
Merged

Fig. 5

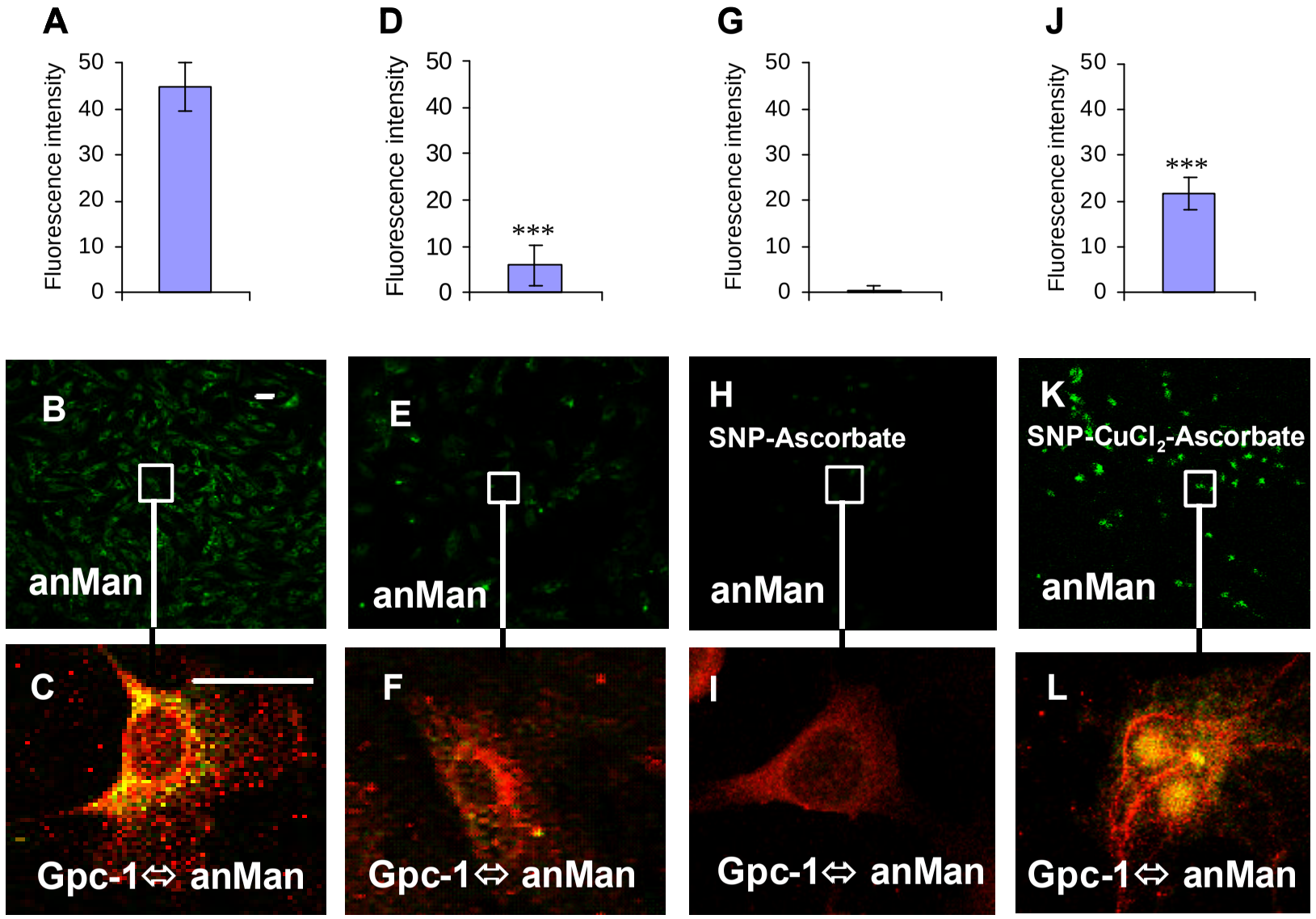
A
D
G
J
Fluorescence intensity
50
40
30
20
10
0
***
B
E
H
SNP-Ascorbate
K
SNP-CuCl2-Ascorbate
anMan
C
F
I
L
Gpc-1⇔ anMan

Fig. 6

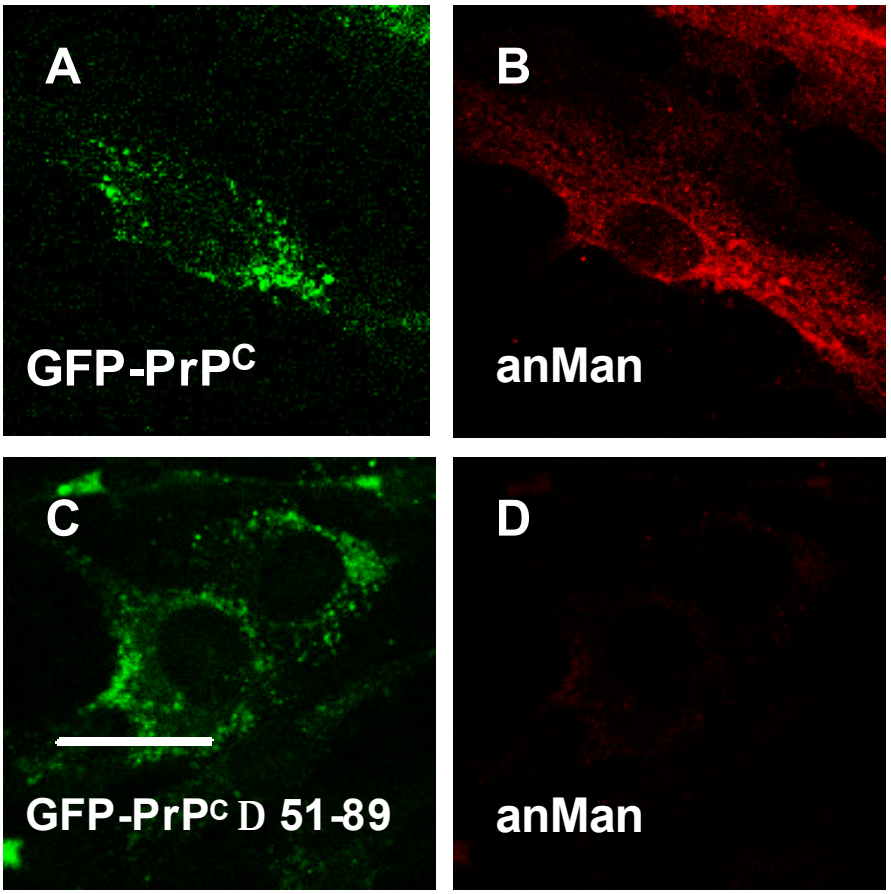

Fig. 7