Assigning functions to distinct regions of the N-terminus of the prion protein that are involved in its copper-stimulated, clathrin-dependent endocytosis

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Summary

The cellular prion protein (PrP^C) is essential for the pathogenesis and transmission of prion diseases. Although PrP^C is known to be located in detergent-insoluble lipid rafts at the surface of neuronal cells, the mechanism of its internalisation is unclear, with both raft/caveolae-based and clathrin-mediated processes being proposed. We have investigated the mechanism of copper-induced internalisation of PrP^C in neuronal cells by immunofluorescence microscopy, surface biotinylation assays and buoyant sucrose density gradient centrifugation in the presence of Triton X-100. Clathrin-mediated endocytosis was selectively blocked with tyrphostin A23, which disrupts the interaction between tyrosine motifs in the cytosolic domains of integral membrane proteins and the adaptor complex AP2, and a dominant-negative mutant of the adaptor protein AP180. Both these agents inhibited the copper-induced endocytosis of PrP^C. Copper caused PrP^C to move laterally out of detergent-insoluble lipid rafts into detergent-soluble regions of the plasma membrane. Using mutants of PrP^C that lack either the octapeptide repeats or the N-terminal polybasic region, and a construct with a transmembrane anchor, we show that copper binding to the octapeptide repeats promotes dissociation of PrP^C from lipid rafts, whereas the N-terminal polybasic region mediates its interaction with a transmembrane adaptor protein that engages the clathrin endocytic machinery. Our results provide an experimental basis for reconciling the apparently contradictory observations that the prion protein undergoes clathrin-dependent endocytosis despite being localised in lipid rafts. In addition, we have been able to assign distinct functions in the endocytic process to separate regions of the protein.

Key words: Prion protein, Copper, Lipid rafts, Clathrin, Endocytosis

Introduction

The prion protein (PrP) is the causative agent of the transmissible spongiform encephalopathies (TSEs), such as Creutzfeldt-Jakob disease in humans, scrapie in sheep and bovine spongiform encephalopathy (Prusiner, 1998). In these diseases, the normal cellular form of PrP (PrP^C) undergoes a conformational conversion to the infectious form PrP^{Sc}. Although the physiological function of PrP^C is still unclear, the protein has been implicated in copper metabolism and the cellular response to oxidative stress (Roucou et al., 2004; Vassallo and Herms, 2003). PrP^C is attached to the cell surface through a glycosylphosphatidylinositol (GPI) anchor and is localised in cholesterol- and glycosphingolipid-rich lipid rafts (Vey et al., 1996). At the plasma membrane, PrP^C can interact with PrP^{Sc} in the de novo formation of PrP^{Sc} aggregates (Lehmann et al., 1999). Accumulating evidence indicates that the conversion of PrP^C to PrP^{Sc} preferentially occurs within lipid rafts (Baron et al., 2002; Naslavsky et al., 1997; Taraboulos et al., 1995; Vey et al., 1996). A reduction in the level of cell-surface PrP^C through enhanced endocytosis or by shedding of the protein from the membrane (Parkin et al., 2004) might reduce PrP^{Sc} production by limiting the amount of PrP^C substrate available for conversion (Marella et al., 2002). Thus, the elucidation of the mechanisms involved in the endocytosis of PrP^C is central to the normal cell biology of the protein and might be of crucial importance in understanding the pathogenesis of TSEs.

The classical and best-characterised mechanism for the endocytosis of cell-surface proteins is through clathrin-coated pits, with sorting at the cell surface achieved through the direct or indirect binding of the cytoplasmic domains of the protein to clathrin-associated proteins (Kirchhausen, 2000). The adaptor proteins AP2 and AP180 are both major components of clathrin coats. The AP2 heterotetrameric complex binds to phosphoinositides in the membrane, as well as to the cytoplasmic domains of membrane proteins destined for internalisation, and interacts with a range of cytoplasmic proteins including AP180 (Gaidarov and Keen, 1999; Owen et al., 2000). Both AP2 and AP180 bind directly to clathrin and stimulate clathrin cage assembly in vitro (Owen et al., 2000; Ye et al., 2000). However, several GPI-anchored proteins, which lack cytoplasmic domains, appear to undergo endocytosis by a non-clathrin-dependent mechanism (Nichols, 2002; Parton et al., 1994; Pelkmans and Helenius, 2002; Rothberg et al., 1990). Such proteins are internalised through caveolae in a raft-dependent mechanism as shown by the

ability to block this process with cholesterol-binding drugs, such as filipin and methyl-β-cyclodextrin (MβCD), presumably as a result of extraction of cholesterol from the rafts/caveolae leading to a loss of their morphological and functional integrity (Nichols, 2003; Schnitzer et al., 1994). However, the mechanism of PrP^C internalisation is still controversial, as both raft/caveolae-based (Kaneko et al., 1997; Marella et al., 2002; Peters et al., 2003; Vey et al., 1996) and clathrin-dependent processes (Magalhaes et al., 2002; Shyng et al., 1994; Sunyach et al., 2003) have been described (reviewed by Prado et al., 2004). Although other studies have investigated the fate of endocytosed PrP^C, the mechanism of its internalisation was not addressed (Brown and Harris, 2003; Hachiya et al., 2004; Lee et al., 2001).

The N-terminal half of PrP^C contains four octapeptide repeats (PHGG(G/S)WGQ; residues 59-90) that preferentially bind Cu^{2+} ions (Stockel et al., 1998; Viles et al., 1999). The physiological importance of Cu^{2+} binding to the octapeptide repeats is shown by the finding that exposure of neuronal cells to concentrations of Cu^{2+} similar to that estimated for the extracellular spaces of the brain results in the rapid internalisation of PrP^C (Pauly and Harris, 1998; Perera and Hooper, 2001). This metal-dependent endocytosis can be abrogated by deletion of the octapeptide repeats, mutation of the histidine residues of the two central repeats, or an insertional mutation within the repeats that is associated with an inherited form of human prion disease (Perera and Hooper, 2001).

In the present study, we have addressed the mechanism of the Cu^{2+}-induced internalisation of mammalian PrP^C in neuronal cells and investigated the role of discrete regions of the N-terminus of the protein in this process. Both tyrphostin A23 and a dominant-negative mutant of the adaptor protein AP180 that selectively inhibit clathrin-mediated endocytosis blocked the endocytosis of PrP^C and transferrin but had no effect on the internalisation of ganglioside GM1. Using both buoyant sucrose density gradient centrifugation in the presence of Triton X-100 and immunofluorescence microscopy, we show that cell-surface PrP^C is normally present in detergent-insoluble lipid rafts but, following exposure to Cu^{2+}, a significant proportion of the protein re-localises to detergent-soluble regions of the plasma membrane. Furthermore, by using mutants of PrP, we show that Cu^{2+} binding to the octapeptide repeats is required to dissociate PrP^C from lipid rafts whereas the N-terminal polybasic region is required for its clathrin-mediated endocytosis.

Materials and Methods

PrP constructs and cell culture

Insertion of the coding sequence of murine PrP containing a 3F4 epitope tag into pIRESneo (BD Biosciences Clontech) and the generation of the PrP-Δoct, PrP-ΔN and PrP-CTM constructs have been reported previously (Perera and Hooper, 2001; Walmsley et al., 2001) (E. T. Parkin, N.T.W. and N.M.H., unpublished). For stable transfection of the cDNA encoding the PrP constructs, 30 μg DNA was introduced to SH-SY5Y cells by electroporation and selection was performed in normal growth medium containing 500 μg/ml neomycin selection antibiotic (Gibco BRL). The cDNAs encoding AP180-C in pCMVmyc and AP180-N in pcDNA4 were a gift from H. T. McMahon (MRC Laboratory of Molecular Biology, Cambridge, UK). Cells were incubated with the vector in the presence of Lipofectamine 2000 (Invitrogen) for 24 hours. Transfected cells were detected using an anti-Myc antibody (Sigma-Aldrich) for AP180-C, or an anti-AP180 antibody (Santa Cruz Biotechnology). SH-SY5Y and N2a cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 50 U/ml penicillin and 0.1 mg/ml streptomycin. Cells were maintained in a humidified incubator at 37°C with 5% CO_2.

Cell-surface biotinylation endocytosis assay

Cells at confluency were incubated for 1 hour at 4°C with 0.5 mg/ml Biotin sulfo-NHS (Sigma-Aldrich). Where indicated, cells were pre-incubated with tyrphostin A23 or tyrphostin A63 (Merck Biosciences) for 5 minutes at 37°C. Cells were then incubated for 20 minutes at 37°C in OptiMEM (Gibco BRL) in the presence or absence of 100 μM $CuSO_4$ presented as a histidine chelate (Perera and Hooper, 2001). Prior to cell lysis, PrP remaining on the cell surface was removed by digestion with trypsin as described previously (Perera and Hooper, 2001).

Immunoprecipitation

Cell lysates were pre-cleared by incubation for 30 minutes with 0.5% (w/v) protein A-Sepharose. The protein A-Sepharose was pelleted by centrifugation for 1 minute at 13,000 ***g*** and the supernatant removed and incubated overnight with 0.5% (v/v) 3F4 antibody (Signet Laboratories) for the transfected SH-SY5Y cell lysates or SAF32 antibody (Spi-Bio) for the untransfected N2a cell lysates or anti-transferrin receptor antibody H68.4 (Zymed Laboratories). Protein A-Sepharose was added to 0.5% (w/v) to the samples and incubation continued at 37°C for 1 hour. Immunocomplexes were pelleted at 13,000 ***g*** for 1 minute and the pellet washed three times with 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS and 1% (v/v) Nonidet P-40.

SDS PAGE and western blot analysis

Immunoprecipitated biotinylated complexes were mixed with dissociation buffer (125 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 20% (v/v) glycerol, 100 mM dithiothreitol, Bromophenol Blue) and boiled for 5 minutes. Proteins were resolved by electrophoresis through 14.5% or 10% polyacrylamide gels for PrP or the transferrin receptor, respectively, and then transferred to Hybond-P polyvinylidene difluoride membrane. The membrane was blocked for 1 hour in phosphate-buffered saline (PBS; 1.5 mM KH_2PO_4, 2.7 mM Na_2HPO_4, 150 mM NaCl, pH 7.4) containing 5% (w/v) dried milk powder and 0.1% (v/v) Tween-20, followed by incubation with peroxidase-conjugated streptavidin [1:1000 dilution in PBS containing 0.1% (v/v) Tween-20] for 1 hour. Bound peroxidase conjugates were visualised using an enhanced chemiluminescence detection system (Amersham Biosciences). Flotillin-1 and transferrin receptor were detected using an anti-flotillin-1 antibody (BD Biosciences Pharmingen) and antibody H68.4, respectively, with a peroxidase-conjugated rabbit anti-mouse secondary antibody (Sigma-Aldrich).

Immunofluorescence microscopy

Cells were seeded onto coverslips and grown to 50% confluency. The fate of cell-surface PrP^C was monitored by pre-labelling cells with antibody 3F4 for 30 minutes at 4°C. For endocytosis experiments, cells were pre-incubated with 500 μM tyrphostin A23 and then incubated for 20 minutes at 37°C in OptiMEM in the presence or absence of 100 μM $CuSO_4$ and tyrphostin A23. During this time, cells were also incubated where indicated with 5 μl of a 5 mg/ml solution of Texas-Red-conjugated transferrin (Molecular Probes) or 10 μl of a 10 mg/ml tetramethylrhodamine-labelled 10,000 MW fixable dextran (Molecular Probes). Where required, cells were permeablised in PBS containing 0.1% Triton X-100, fixed with 4% (v/v)

paraformaldehyde/0.1% (v/v) glutaraldehyde in PBS for 15 minutes, and blocked overnight in PBS containing 3% (v/v) goat serum (Sigma-Aldrich). Finally, coverslips were incubated with the appropriate fluorescent-probe-conjugated secondary antibodies (Molecular Probes) for 1 hour and mounted on slides using fluoromount G mounting medium (SouthernBiotech). Individual cells were visualised using a DeltaVision Optical Restoration Microscopy System (Applied Precision). Data were collected from 30-40 0.1 μm thick optical sections, and 3D datasets were deconvolved using the softWoRx programme (Applied Precision). The presented images represent individual Z-slices with only one Z-slice per image used for quantitation purposes. Analysis of cell-surface PrP^C staining was performed using ImageJ (http://rsb.info.nih.gov/ij/) to measure the intensity of fluorescence around the cell membrane. This was plotted as Pixel Intensity versus Distance around the cell using Microsoft Excel, and then the percentage of cell surface with detectable staining was calculated from multiple images.

Triton X-100 and MβCD treatments

In the Triton X-100 extraction experiments, antibody 3F4-labelled cells were incubated at 4°C for 10 minutes with PBS containing 1% Triton X-100 prior to paraformaldehyde and then incubated with Alexa488-conjugated rabbit anti-mouse antibody. For disruption of rafts by cholesterol extraction, cells at confluency were incubated with 1 mM MβCD for 1 hour at 37°C.

BODIPY FL C_5-ganglioside GM1 endocytosis

Cells, unlabelled with antibody 3F4 but pre-incubated with tyrphostin A23 or MβCD, were incubated with 5 μM BODIPY FL C_5-ganglioside GM1 (Molecular Probes) in the presence or absence of 500 μM tyrphostin A23 or 1 mM MβCD for 20 minutes at 37°C.

Lipid raft isolation

Harvested cells were resuspended in 2 ml MES-buffered saline (MBS; 25 mM Mes, 150 mM NaCl, pH 6.5) containing 1% Triton X-100 and homogenised by passing 15 times through a Luer 21-gauge needle. After centrifugation at 500 ***g*** for 5 minutes, the supernatant was made up to 40% sucrose by adding an equal volume of 80% sucrose in MBS. A 1 ml aliquot of the sample was placed beneath a discontinuous gradient of sucrose consisting of 3 ml of 30% sucrose and 1 ml of 5% sucrose, both in MBS. The samples were then centrifuged at 140,000 ***g*** in an SW-55 rotor (Beckman Coulter) for 18 hours at 4°C. The sucrose gradients were harvested in 0.5 ml fractions from the base of the gradient and the distribution of proteins monitored by western blot analysis of the individual fractions.

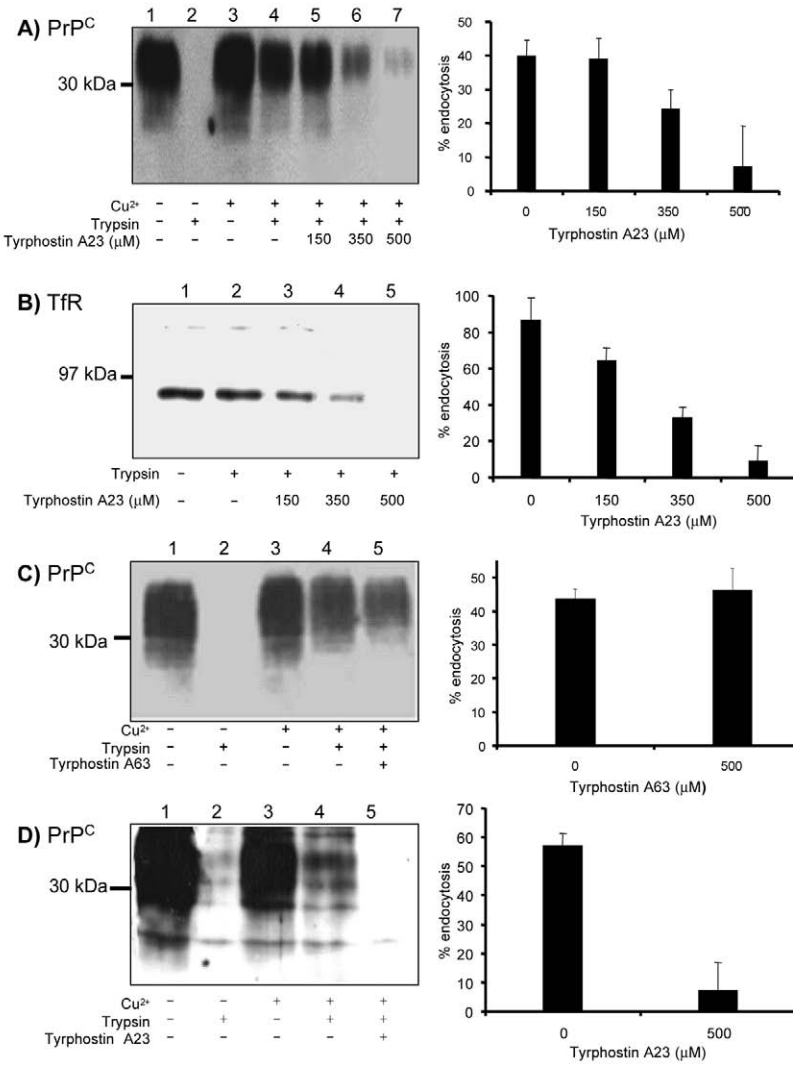

Fig. 1. The copper-stimulated endocytosis of PrP^C is blocked by tyrphostin A23. (A) SH-SY5Y cells expressing PrP^C were surface biotinylated and then either untreated or treated with tyrphostin A23 in the presence or absence of 100 μM Cu^{2+}. Prior to lysis, the cells were incubated with trypsin to digest cell-surface PrP^C. Cells were then lysed, and total PrP^C was immunoprecipitated from the sample using antibody 3F4 and then subjected to western blot analysis. The biotin-labelled PrP^C fraction was detected with peroxidase-conjugated streptavidin. (B) The same samples from (A) were also immunoprecipitated using an anti-transferrin receptor antibody. (C) Cells were treated with tyrphostin A63 and processed as in (A). (D) Biotin-labelled N2a cells were processed as described in (A), but PrP^C was immunoprecipitated using the antibody SAF-32. Densitometric analysis (mean±s.e.m.) of multiple blots from three separate experiments is shown.

Results

Tyrphostin A23 blocks the Cu^{2+}-induced endocytosis of PrP^C

Previously we have shown that PrP^C in the human neuroblastoma SH-SY5Y cell line undergoes rapid Cu^{2+}-mediated endocytosis (Perera and Hooper, 2001). In order to investigate the mechanism of this endocytosis, SH-SY5Y cells stably transfected with the cDNA encoding murine PrP^C were incubated in the presence of tyrphostin A23 that has been shown to inhibit selectively clathrin-mediated endocytosis (Banbury et al., 2003; Crump et al., 1998). In order to distinguish surface protein that was endocytosed from protein either already endocytosed or in the secretory pathway, surface proteins were biotinylated with a cell-impermeant reagent prior to incubation of the cells in serum-free medium that contained Cu^{2+} presented as a histidine chelate (Perera and Hooper, 2001). After incubation of the cells at 37°C for 20 minutes,

residual surface PrP^C was removed by trypsin treatment prior to lysis of the cells. Any surface biotinylated PrP^C that was endocytosed during the course of the experiment was protected from trypsin digestion. In the absence of Cu^{2+}, no endocytosis of PrP^C was observed (Fig. 1A, lane 2), whereas 100 μM Cu^{2+} caused the internalisation of the biotinylated protein (Fig. 1A, lane 4), consistent with our previous results (Perera and Hooper, 2001). Tyrphostin A23 blocked this Cu^{2+}-mediated endocytosis of cell-surface PrP^C in a dose-dependent manner (Fig. 1A, lanes 5-7). Tyrphostin A23 also blocked the Zn^{2+}-stimulated endocytosis of PrP^C (data not shown).

The endocytosis of the transferrin receptor in the SH-SY5Y cells was also inhibited in a dose-dependent manner by tyrphostin A23 (Fig. 1B), consistent with this protein being endocytosed through clathrin-coated pits (Dautry-Varsat et al., 1983). The specificity of the blockade of clathrin-mediated endocytosis by tyrphostin A23 was shown by the observation that the structurally related tyrphostin A63, which does not inhibit transferrin receptor internalisation (Banbury et al., 2003), failed to block the Cu^{2+}-mediated endocytosis of PrP^C (Fig. 1C). To ascertain that endocytosis of PrP^C by a clathrin-mediated mechanism was not an artefact of the expression of PrP in the SH-SY5Y cells, the effect of tyrphostin A23 on the internalisation of endogenous PrP^C in the mouse neuroblastoma N2a cell line was examined (Fig. 1D). As in the SH-SY5Y cells, tyrphostin A23 blocked the Cu^{2+}-mediated endocytosis of cell-surface PrP^C in the N2a cells.

These results were confirmed using immunofluorescence microscopy (Fig. 2A). To monitor directly the fate of cell-surface PrP^C during the course of the experiment, SH-SY5Y cells were prelabelled with antibody 3F4 at 4°C in the absence of Cu^{2+}. Following incubation with Cu^{2+} at 37°C, the cells were fixed, permeabilised and reacted with fluorescently labelled secondary antibody to visualise the distribution of antibody-tagged PrP^C. Prior to incubation of the cells with Cu^{2+}, PrP^C was localised predominantly at the cell surface. Following incubation of the cells with Cu^{2+}, a significant proportion of intracellular staining for PrP^C was observed. However, in the presence of tyrphostin A23, very little intracellular staining was observed upon incubation of the cells with Cu^{2+}; the majority of the PrP^C was localised at the cell surface, indicating that tyrphostin A23 was blocking the internalisation of PrP^C. Similarly, tyrphostin A23 blocked the constitutive endocytosis of transferrin in the SH-SY5Y cells (Fig. 2A).

In order to ensure that tyrphostin A23 had no effect on raft-based endocytosis, we examined the endocytosis of a fluorescently labelled ganglioside GM1 (Fig. 2B) that is internalised by a raft-based mechanism (Cobbold et al., 2003). Although tyrphostin A23 had no effect on the endocytosis of BODIPY FL C_5-ganglioside GM1, the cholesterol-binding drug MβCD completely blocked its endocytosis, consistent with it being internalised by a cholesterol-dependent raft-based mechanism in the SH-SY5Y cells.

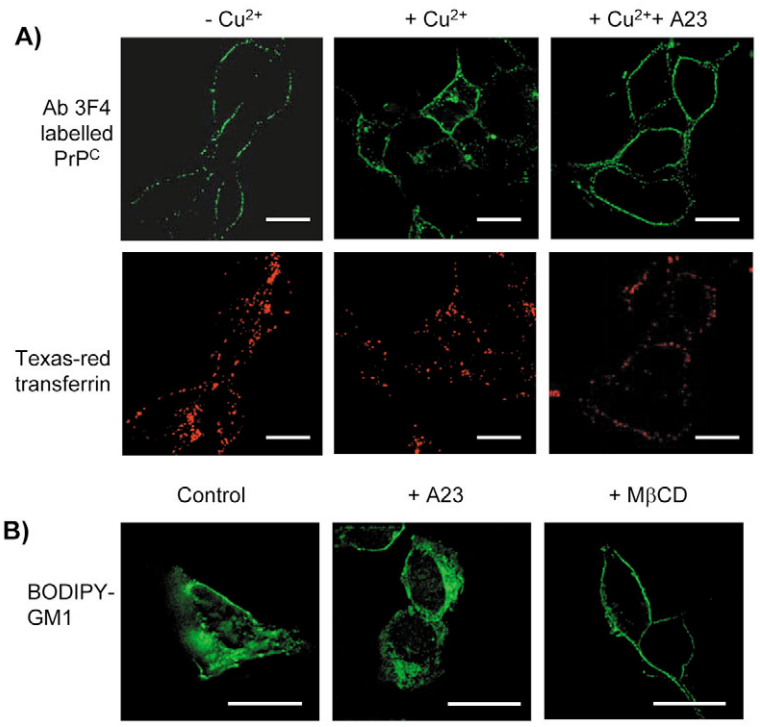

Fig. 2. Tyrphostin A23 blocks PrP^C and transferrin endocytosis but has no effect on ganglioside GM1 endocytosis. (A) SH-SY5Y cells stably expressing PrP^C were seeded onto glass coverslips and grown to 50% confluency. Cells were then pre-incubated with antibody 3F4 at a dilution of 1:1000 in PBS for 30 minutes at 4°C, washed three times in PBS and then incubated for 20 minutes at 37°C in OptiMEM in the absence of Cu^{2+}, in the presence of 100 μM Cu^{2+} or in the presence of Cu^{2+} and 500 μM tyrphostin A23. Cells were also incubated with Texas-Red-conjugated transferrin at a dilution of 1:1000. (B) Cells were incubated with BODIPY FL C_5-ganglioside GM1 for 20 minutes in the presence of either 500 μM tyrphostin A23 or 1 mM MβCD. Cells were fixed, incubated with Alexa488-conjugated rabbit anti-mouse antibody and viewed using a DeltaVision Optical Restoration Microscopy System. Images are representative of three individual experiments. Bars, 10 μm.

A dominant-negative form of AP180 blocks the Cu^{2+}-induced endocytosis of PrP

To confirm that PrP^C was being endocytosed by a clathrin-mediated mechanism, SH-SY5Y cells were cotransfected with a construct (AP180-C) of the adaptor protein AP180. This dominant-negative form of AP180 has been shown to inhibit the uptake of epidermal growth factor and transferrin through disrupting the formation of clathrin-coated pits (Ford et al., 2001). Efficient transient transfection of AP180-C into the SH-SY5Y cells was confirmed by immunofluorescence microscopy using an antibody against the Myc tag on the C-terminus of the protein (Fig. 3A). AP180-C completely blocked the Cu^{2+}-mediated endocytosis of PrP^C and the internalisation of transferrin (Fig. 3A). By contrast, when cells were cotransfected with the N-terminal domain of AP180 (AP180-N), no such inhibition of endocytosis was observed (Fig. 3A), in agreement with previous results (Ford et al., 2001). To provide a quantitative measure of these observations in a population of cells, the percentage of AP180-C- and AP180-N-transfected cells showing endocytosis of transferrin and PrP^C relative to untransfected control cells is given (Fig. 3B). In order to guard against the

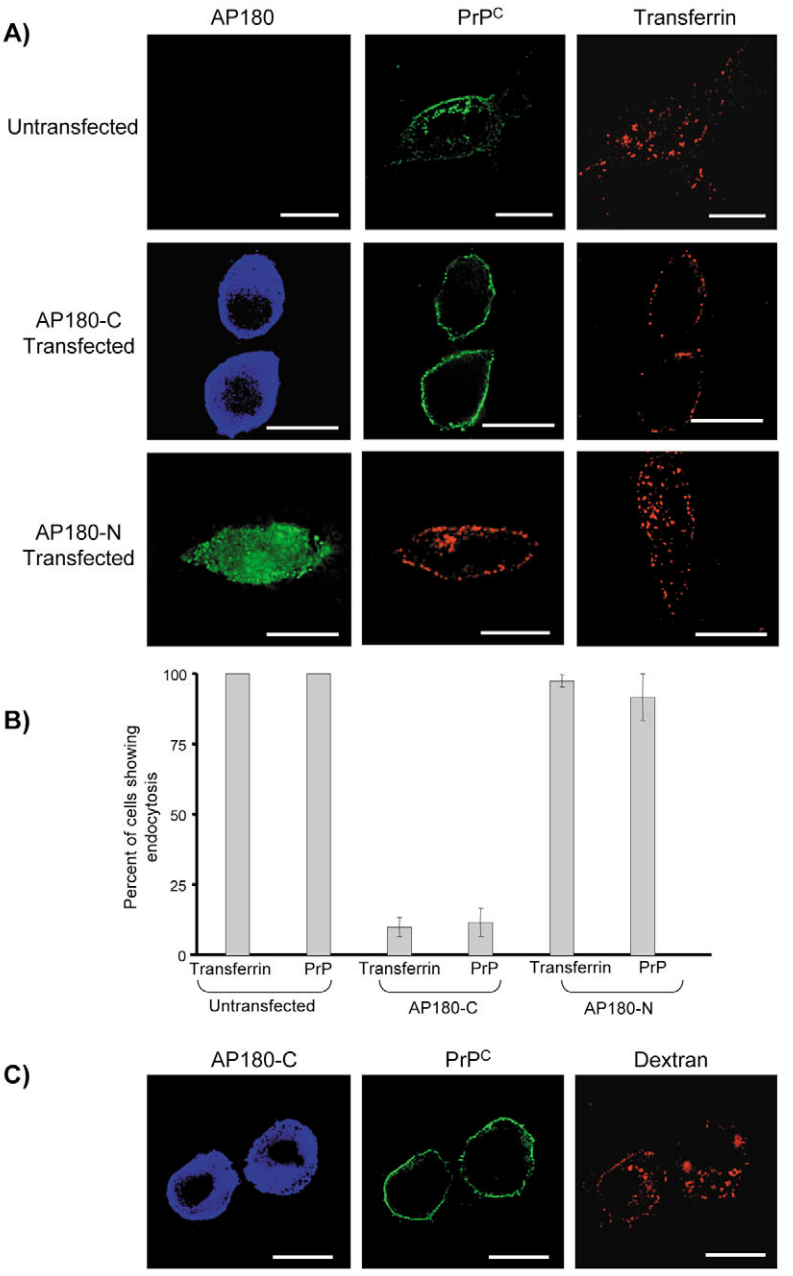

Fig. 3. Overexpression of AP180-C inhibits the Cu^{2+}-stimulated endocytosis of PrP^C. SH-SY5Y cells stably expressing PrP^C were seeded onto coverslips and transiently transfected with either an expression plasmid encoding Myc-tagged AP180-C or an expression plasmid encoding AP180-N. After 24 hours, cells were processed as described in Fig. 2, with AP180-C detected using a polyclonal anti-Myc primary antibody and Marina Blue goat anti-rabbit IgG secondary antibody, and AP180-N detected using a polyclonal anti-AP180 primary antibody and Alexa488-conjugated donkey anti-goat IgG secondary antibody. In AP180-N-transfected cells, 3F4-labelled PrP was detected using an Alexa594-conjugated rabbit anti-mouse IgG secondary antibody. (A) Cells expressing AP180-C are unable to endocytose either transferrin or PrP^C, whereas endocytosis is unaffected in AP180-N-expressing cells. (B) Quantification of (A). Results are the mean±s.e.m. of four individual experiments, in each of which 30 cells were visualised to determine their ability to endocytose transferrin and PrP^C. The number of AP180-C- and AP180-N-transfected cells showing endocytosis is shown as a percentage of the number of untransfected cells showing endocytosis. (C) AP180-C cells in which clathrin-mediated endocytosis is blocked are not senescent, as shown by the uptake of dextran. Bars, 10 μm.

possibility that AP180-C-transfected cells could not endocytose PrP^C because they were senescent and thus not endocytosing by any mechanism at all, we looked at the uptake of dextran in these cells. As can clearly be seen (Fig. 3C), the AP180-C-transfected cells (n=30) in which PrP^C internalisation is blocked are still able to take up dextran from the medium. Taken together, these data unequivocally show that, in two neuronal cell lines, PrP^C is endocytosed through a clathrin-dependent mechanism and not through a raft-based mechanism.

Cu^{2+} causes cell-surface PrP^C to exit detergent-insoluble rafts and translocate to detergent-soluble regions of the plasma membrane

PrP^C in the SH-SY5Y cells is localised in detergent-insoluble lipid rafts (Perera and Hooper, 1999; Walmsley et al., 2003) and yet is endocytosed by a clathrin-dependent mechanism (see above). Either the whole detergent-insoluble raft containing PrP^C could be internalised or PrP^C moves out of the raft into non-raft regions of the membrane prior to endocytosis. To distinguish between these two possibilities, two complementary approaches were used: (1) cell-surface immunofluorescence and (2) buoyant sucrose density gradient centrifugation in the presence of Triton X-100.

For the immunofluorescence experiments, cells expressing PrP^C were pre-incubated with antibody 3F4 at 4°C to label cell-surface PrP^C, then incubated at 37°C in the absence or presence of Cu^{2+}. These experiments were performed in the presence of tyrphostin A23 to block endocytosis. To determine the distribution of PrP^C between detergent-insoluble rafts and the detergent-soluble regions of the membrane, the cells were incubated with 1% Triton X-100 prior to fixation and immunofluorescence microscopy (Fig. 4A). In the absence of Cu^{2+}, PrP^C had a punctate appearance on the cell surface, consistent with that observed for other raft-associated proteins (Ledesma et al., 1999; Parkin et al., 2003; Zeng et al., 2003b). This punctate appearance remained unaltered when cells incubated in the absence of copper were subsequently treated with Triton X-100 prior to fixation, as the detergent only solubilised the non-raft regions of the membrane. Following incubation of the cells with Cu^{2+}, PrP^C had a more even distribution over the cell surface. When the Cu^{2+}-treated cells were incubated with Triton X-100 prior to fixation, the surface distribution of PrP^C returned to a more punctate appearance, as the PrP^C outside of raft regions was solubilised by the detergent. To provide a quantitative measure of this, the percentage of the surface area covered by PrP^C was determined as described in the Materials and Methods from the fluorescence images (Fig. 4B). The percentage of the cell surface with PrP^C staining in the

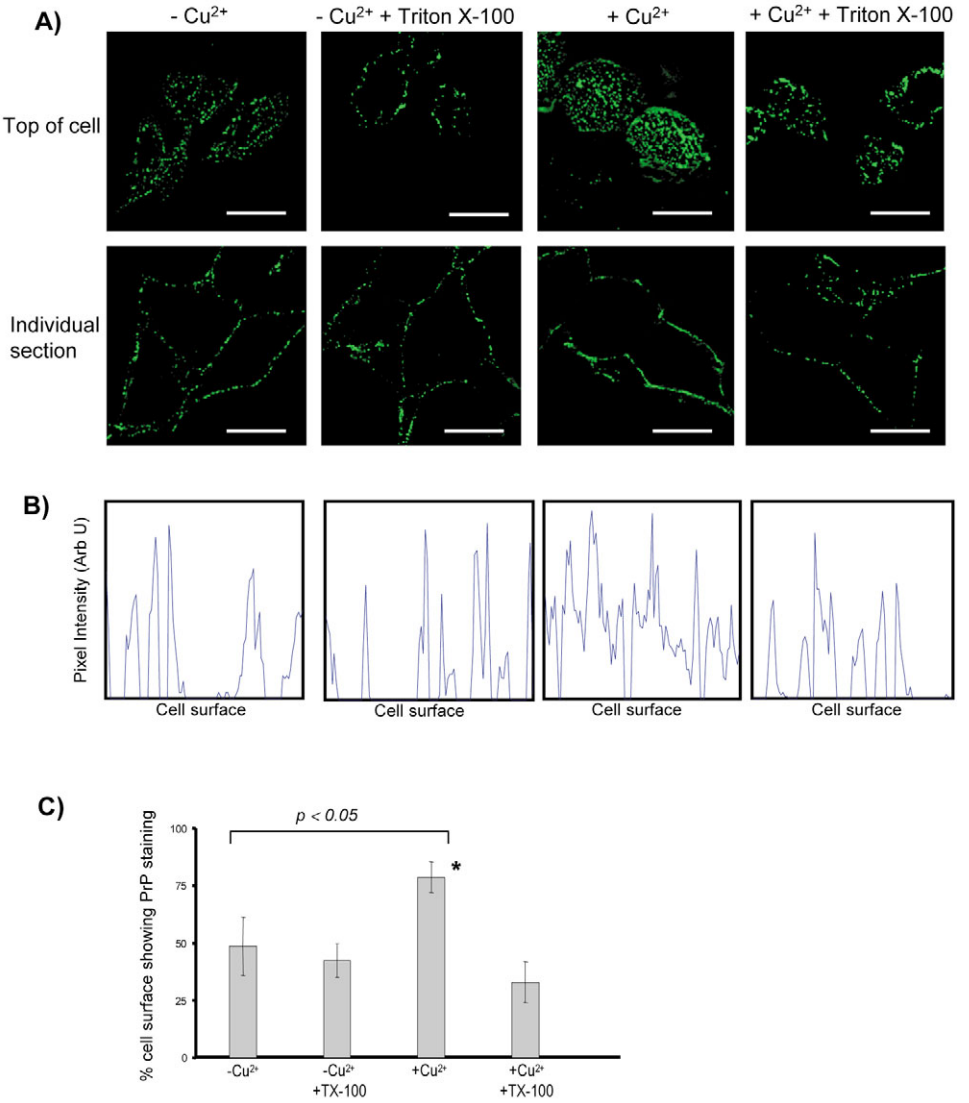

Fig. 4. Effect of Cu^{2+} on the cell-surface distribution of PrP^C. SH-SY5Y cells stably expressing PrP^C were seeded onto glass coverslips and grown to 50% confluency. Cells were then pre-incubated with antibody 3F4 at a dilution of 1:1000 in PBS for 30 minutes at 4°C, washed three times in PBS and then incubated for 20 minutes at 37°C in OptiMEM in the absence or presence of 100 μM Cu^{2+} along with 500 μM tyrphostin A23. Where indicated, cells were incubated at 4°C for 10 minutes with PBS containing 1% Triton X-100 prior to fixation. Cells were fixed, incubated with Alexa488-conjugated secondary antibody and viewed using a DeltaVision Optical Restoration Microscopy System. (A) Images were taken of ten Z-slices from the top of the cell, and of an individual Z-slice from the middle of the cell. Bars, 10 μm. (B) Cell-surface distribution of PrP was measured using ImageJ as described in the Materials and Methods. Average fluorescent intensities per unit length of membrane were as follows: $–Cu^{2+}$, 15.14 units; $–Cu^{2+}$ + Triton X-100, 13.46 units; $+Cu^{2+}$, 16.22 units; $+Cu^{2+}$ + Triton X-100, 8.58 units. (C) The percentage of the cell surface stained for PrP^C was determined from an individual Z-slice image in panel (A). Results are the mean±s.e.m. of three individual experiments, in each of which 30 cells were measured. Statistical differences, using Student's *t*-test (n=90), with probability values of P<0.05 were taken as significant.

absence of Cu^{2+} with or without subsequent Triton X-100 treatment, and in the presence of Cu^{2+} with or without Triton X-100 treatment, respectively, was then represented graphically (Fig. 4C). In the absence of Cu^{2+}, 49±13% of the cell surface was covered by PrP^C and this remained essentially unchanged at 43±7% following treatment with Triton X-100. By contrast, the percentage of the cell surface with PrP^C staining increased to 79±7% upon incubation of the cells with Cu^{2+} and this decreased to 33±9% upon treatment of the cells with Triton X-100.

For the approach using buoyant sucrose density gradient centrifugation in the presence of detergent, cells were first surface biotinylated and then incubated with Cu^{2+} in the presence of tyrphostin A23 to block endocytosis. The cells were homogenised in the presence of Triton X-100 and subjected to buoyant sucrose density gradient centrifugation (Fig. 5). Rafts, as shown by the position of the raft marker protein flotillin-1, migrated from the 40% sucrose layer to the 5%/30% interface of the gradient owing to their high ratio of lipid to protein, whereas non-raft proteins, as shown by the position of the transferrin receptor, remained at the base of the centrifuge tube. In cells not incubated with Cu^{2+}, biotinylated PrP^C was located exclusively at the 5%/30% sucrose interface (Fig. 5A), consistent with the protein residing in detergent-insoluble rafts at the cell surface under basal conditions. However, following incubation of the cells with Cu^{2+}, the majority of the biotinylated PrP^C was detected in the detergent-soluble region of the sucrose gradient (Fig. 5B), indicating that it was no longer resident in the detergent-insoluble rafts. This relocation of PrP^C to detergent-soluble regions of the membrane in the presence of Cu^{2+} was not due to non-specific disruption of the rafts, as the raft-associated protein flotillin-1 was detected exclusively at the 5%/30% sucrose interface in both the absence or presence of Cu^{2+} (Fig. 5A,B). Together, these data suggest that, on exposure of the cells to Cu^{2+}, PrP^C moves out of the detergent-insoluble lipid rafts into detergent-soluble regions of the plasma membrane prior to clathrin-mediated endocytosis.

Binding of Cu^{2+} to the octapeptide repeats dissociates PrP^C from lipid rafts, whereas the N-terminal polybasic region mediates its internalisation

Previously, we have shown that a mutant of PrP lacking the

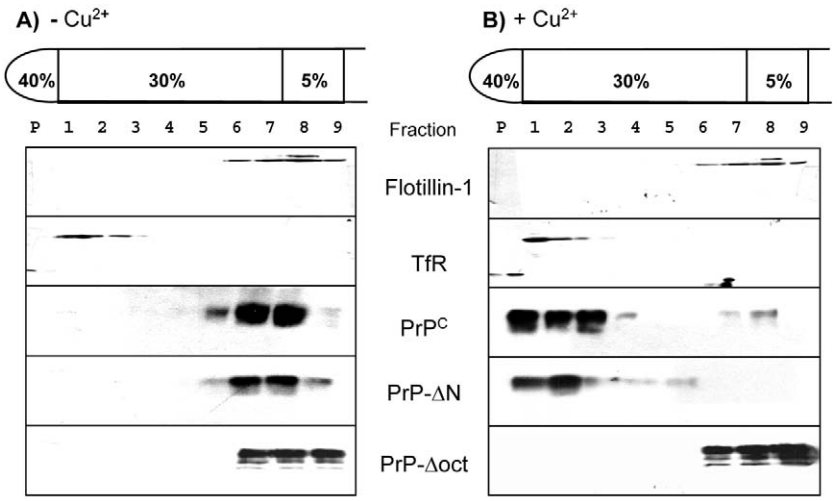

Fig. 5. Effect of Cu^{2+} on the distribution of PrP in detergent-insoluble rafts. SH-SY5Y cells expressing PrP^C, PrP-ΔN or PrP-Δoct were surface biotinylated and incubated in the absence or presence of 100 μM Cu^{2+} along with 500 μM tyrphostin A23. Cells were homogenised in the presence of 1% (v/v) Triton X-100 and subjected to buoyant sucrose density gradient centrifugation. PrP constructs were immunoprecipitated from equal volumes of each gradient fraction using 3F4 and subjected to western blotting. The biotin-labelled PrP^C fraction was detected with peroxidase-conjugated streptavidin. Flotillin-1 and transferrin receptor (TfR) were detected using anti-flotillin-1 and H68.4 antibodies, respectively.

octapeptide repeats (PrP-Δoct; Fig. 6A) fails to be endocytosed upon exposure of cells to Cu^{2+} (Perera and Hooper, 2001). Another mutant, PrP-ΔN (Fig. 6A), which lacks the four residues (KKRP) at the N-terminus of the mature protein, also fails to undergo Cu^{2+}-mediated endocytosis (Fig. 6B). In an attempt to understand the mechanism by which PrP^C moves laterally out of rafts prior to clathrin-mediated endocytosis, we examined the surface distribution of PrP-Δoct and PrP-ΔN by immunofluorescence microscopy before Cu^{2+} treatment, after Cu^{2+} treatment and following incubation with Triton X-100. Both mutants had a punctate appearance on the cell surface in the absence of Cu^{2+} (Fig. 6C,D) consistent with their localisation in rafts. However, upon incubation of the cells with Cu^{2+}, only PrP-ΔN and not PrP-Δoct displayed a more even distribution on the cell surface that was reversed upon treatment with Triton X-100 (Fig. 6C,D). Quantification of the amount of cell-surface staining for PrP-ΔN and PrP-Δoct clearly indicated that Cu^{2+} increased the amount of cell-surface staining for PrP-ΔN but had no significant effect on the amount of cell-surface staining of PrP-Δoct (Fig. 6E).

The raft association of PrP-Δoct and PrP-ΔN following incubation of the cells with Cu^{2+} was also examined by buoyant sucrose density gradient centrifugation in the presence of Triton X-100 (Fig. 5). In the absence of Cu^{2+}, both mutants were localised exclusively in the raft fractions of the sucrose gradient (Fig. 5A). However, following incubation of the cells with Cu^{2+}, PrP-ΔN, like PrP^C, was present predominantly in the soluble region at the bottom of the sucrose gradient (Fig. 5B), whereas PrP-Δoct was still localised in the raft fractions near the top of the gradient (Fig. 5B). Together, these data indicate that binding of Cu^{2+} to the octapeptide repeats is required to displace PrP^C from the detergent-insoluble rafts into the detergent-soluble region of the cell membrane and that the basic residues at the extreme N-terminus of PrP^C are required to mediate its internalisation.

Displacement of PrP^C from lipid rafts triggers its endocytosis

The above data suggest that Cu^{2+} actually promotes the endocytosis of PrP^C by dissociating the protein from another component within rafts, thereby enabling it to interact through its N-terminal polybasic region with the clathrin endocytosis machinery. We explored this hypothesis in two ways. First, we reasoned that disrupting rafts would result in the endocytosis of PrP^C even in the absence of Cu^{2+}. To this end, cells were incubated with MβCD to disrupt the rafts and the endocytosis of PrP^C was examined (Fig. 7A). As predicted, in the absence of Cu^{2+}, MβCD treatment caused an increase in the endocytosis of PrP^C, a process that was still inhibited by tyrphostin A23. This increased endocytosis of PrP^C was relatively modest, as MβCD also promotes the shedding of cell-surface PrP^C (Parkin et al., 2004). MβCD had no significant effect on the endocytosis of the transferrin receptor under these conditions (Fig. 7A).

Second, we utilised a construct, PrP-CTM (Fig. 5A), in which the GPI anchor attachment signal in PrP^C is replaced with the transmembrane and cytosolic domains from angiotensin-converting enzyme, which lacks a known endocytosis signal (Walmsley et al., 2001). This transmembrane anchored form of PrP when expressed in SH-SY5Y cells displayed a less-punctate surface distribution than PrP^C (Fig. 7B), suggesting that it was not completely localised in rafts at the cell surface. This was confirmed following incubation of the cells with either Triton X-100 or MβCD. Whereas solubilisation of the non-raft regions of the plasma membrane with Triton X-100 had little effect on the punctate appearance of PrP^C, the surface staining of PrP-CTM was significantly reduced (Fig. 7B). When the cholesterol-binding agent MβCD was used to disrupt rafts, the punctuate distribution of PrP^C changed to a more diffuse staining pattern, whereas the diffuse staining pattern of PrP-CTM at the cell surface did not change (Fig. 7B). These data indicate that PrP-CTM is not localised in lipid rafts at the cell surface. Consistent with our hypothesis, biotinylated PrP-CTM was rapidly endocytosed from the cell surface in the absence of Cu^{2+} (Fig. 7C). This Cu^{2+}-independent internalisation of PrP-CTM was blocked by tyrphostin A23 (Fig. 7C), indicating that it was being endocytosed by a clathrin-mediated mechanism.

Discussion

In this study, we show that PrP^C is present in detergent-insoluble lipid rafts at the surface of neuronal cells but that, on

exposure to Cu^{2+}, the protein moves laterally out of the rafts into detergent-soluble regions of the plasma membrane prior to internalisation by clathrin-mediated endocytosis. Furthermore, we show that binding of Cu^{2+} to the octapeptide repeats is required to dissociate PrP^C from the lipid rafts, whereas the polybasic region at the N-terminus of the mature protein is required to mediate its clathrin-dependent internalisation. Our data thus provide an experimental basis for reconciling the apparently contradictory observations that, although localised in lipid rafts, PrP^C undergoes clathrin-dependent endocytosis and, in addition, assigns separate functions to distinct regions of the protein.

We investigated the mechanism of PrP^C endocytosis by the novel approach of selectively blocking clathrin-mediated endocytosis. Tyrphostin A23 has been shown to disrupt specifically the interaction between tyrosine motifs (YXXΦ where Φ represents a bulky hydrophobic residue) in the cytosolic domains of integral membrane proteins and the medium chain (μ) subunits of adaptor complex AP2 that links to the clathrin coat (Banbury et al., 2003). The dominant-negative C-terminal fragment AP180-C blocks clathrin-coated pit formation and inhibited uptake of epidermal growth factor and transferrin, whereas the N-terminal fragment AP180-N had no such inhibitory effect (Ford et al., 2001). The observation that both tyrphostin A23 and AP180-C block the endocytosis of PrP^C provides unequivocal evidence that clathrin-coated pits are involved in the Cu^{2+}-stimulated internalisation of PrP^C. During the course of the present study, Sunyach et al. used electron microscopy to show that PrP^C was constitutively endocytosed in the absence of Cu^{2+} by clathrin-coated pits in primary neurons and N2a cells as

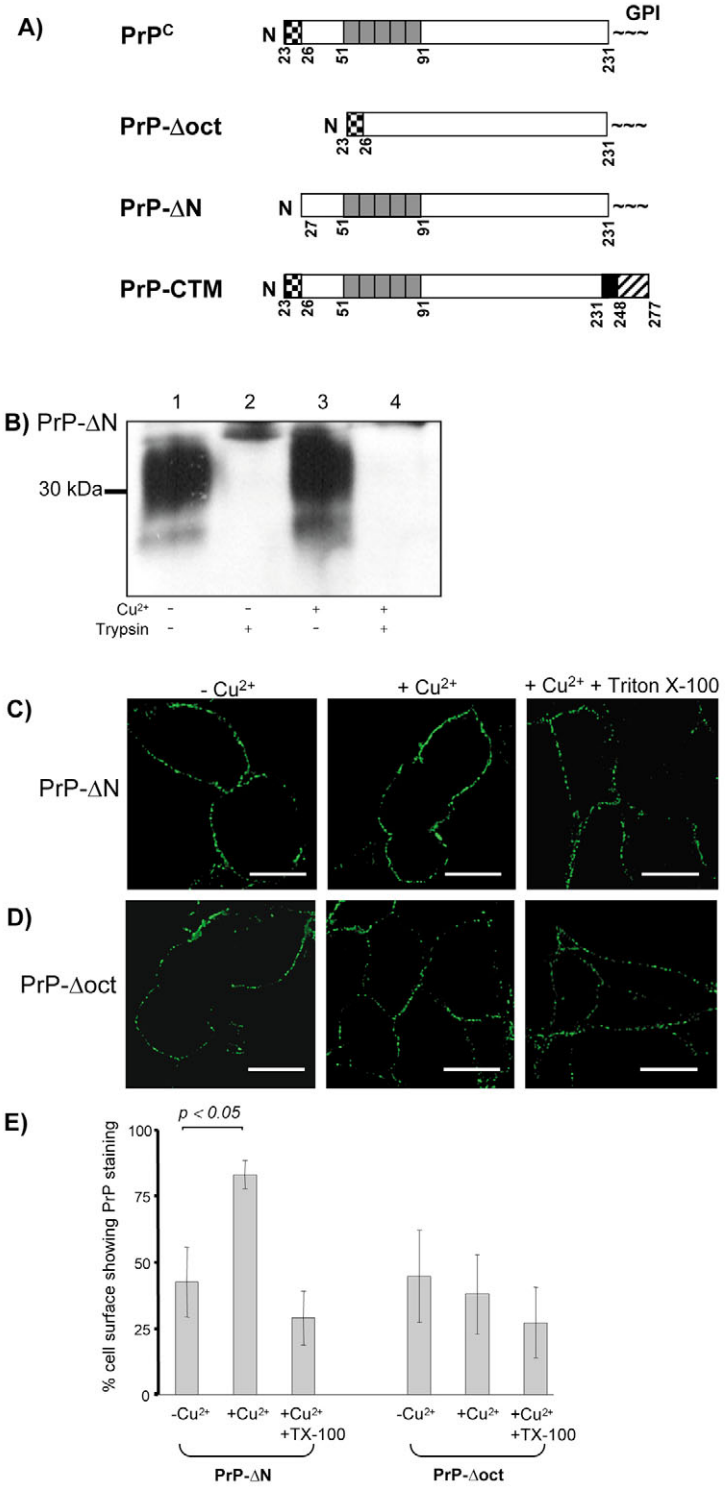

Fig. 6. Effect of Cu^{2+} on the cell-surface distribution of PrP-ΔN and PrP-Δoct. (A) Schematic of the PrP constructs used in this study. PrP^C is shown as the mature, full-length protein (residues 23-231) with its C-terminal GPI anchor, the N-terminal polybasic region (KKRP, residues 23-26, chequered box) and the octapeptide repeats (residues 51-91, shaded). PrP-Δoct lacks the entire octapeptide repeat region and PrP-ΔN lacks the N-terminal polybasic region. In PrP-CTM, the GPI anchor attachment signal is replaced with the transmembrane (filled box) and cytoplasmic domains (cross-hatched) from angiotensin-converting enzyme (Walmsley et al., 2001). (B) SH-SY5Y cells expressing PrP-ΔN were surface biotinylated and then incubated in the absence or presence of 100 μM Cu^{2+}. Prior to lysis, the cells were incubated with trypsin to digest cell-surface PrP. Cells were then lysed, and total PrP was immunoprecipitated from the sample using antibody 3F4 and then subjected to western blot analysis. The biotin-labelled PrP fraction was detected with peroxidase-conjugated streptavidin. SH-SY5Y cells stably expressing (C) PrP-ΔN or (D) PrP-Δoct were seeded onto glass coverslips and grown to 50% confluency. Cells were then pre-incubated with antibody 3F4 at a dilution of 1:1000 in PBS for 30 minutes at 4°C, washed three times in PBS and then incubated for 20 minutes at 37°C in OptiMEM in the absence or presence of 100 μM Cu^{2+} along with 500 μM tyrphostin A23. Where indicated, cells were incubated at 4°C for 10 minutes with PBS containing 1% Triton X-100 prior to fixation. Cells were fixed, incubated with Alexa488-conjugated secondary antibody and viewed using a DeltaVision Optical Restoration Microscopy System. Images are representative of three individual experiments. Bar, 10 μm. Average fluorescent intensity per unit length of membrane for PrP-ΔN and PrP-Δoct were as follows: $-Cu^{2+}$, 11.58 and 10.54 units; $+Cu^{2+}$, 12.61 and 10.10 units; $+Cu^{2+}$ + Triton X-100, 9.09 and 8.81 units, respectively. (E) The percentage of the cell surface stained for PrP-ΔN and PrP-Δoct was determined from the images in (C) and (D), respectively, as described in Fig. 4. Results are the mean±s.e.m. of three individual experiments, in each of which 30 cells were measured. Statistical differences (n=90), using Student's t-test, with probability values of P<0.05 were taken as significant.

a result of its predominant colocalisation with the transferrin receptor rather than with the GPI-anchored Thy-1 (Sunyach et al., 2003). Previously, chicken PrP had been localised to clathrin-coated pits and vesicles by electron microscopy (Shyng et al., 1994).

By contrast, it has been reported that PrP^C is endocytosed by a caveolin-dependent pathway based on the observation that the cholesterol-binding agent filipin prevented its Cu^{2+}-stimulated endocytosis (Marella et al., 2002). However, this conclusion is confused by the observation that filipin also promotes the shedding of PrP^C (Marella et al., 2002; Parkin et al., 2004). Another study concluded that PrP^C is endocytosed through a caveolae-mediated pathway (Peters et al., 2003), although in this case non-neuronal CHO cells were used. As neuronal cells lack caveolin (Gorodinsky and Harris, 1995; Parkin et al., 1997) and morphologically distinguishable caveolae (Shyng et al., 1994), the relevance of these observations to the in vivo situation remains unclear.

In addition to PrP^C, a few other proteins are localised in detergent-insoluble lipid rafts at the cell surface and yet are internalised by a clathrin-dependent mechanism. These include the epidermal growth factor receptor (Mineo et al., 1999) and the anthrax toxin receptor (Abrami et al., 2003). In the latter case, the authors concluded that 'further studies will be required to determine whether the anthrax toxin receptor moves laterally out of rafts before endocytosis' (Abrami et al., 2003). Similarly, both the ganglioside-binding cholera toxin and the glycosphingolipid-binding Shiga toxin are found in detergent-insoluble domains but are internalised by clathrin-coated pits (Sandvig et al., 1989; Shogomori and Futerman, 2001). To rationalise their data, Shogomori and Futerman put forward two models for the mechanism of internalisation of

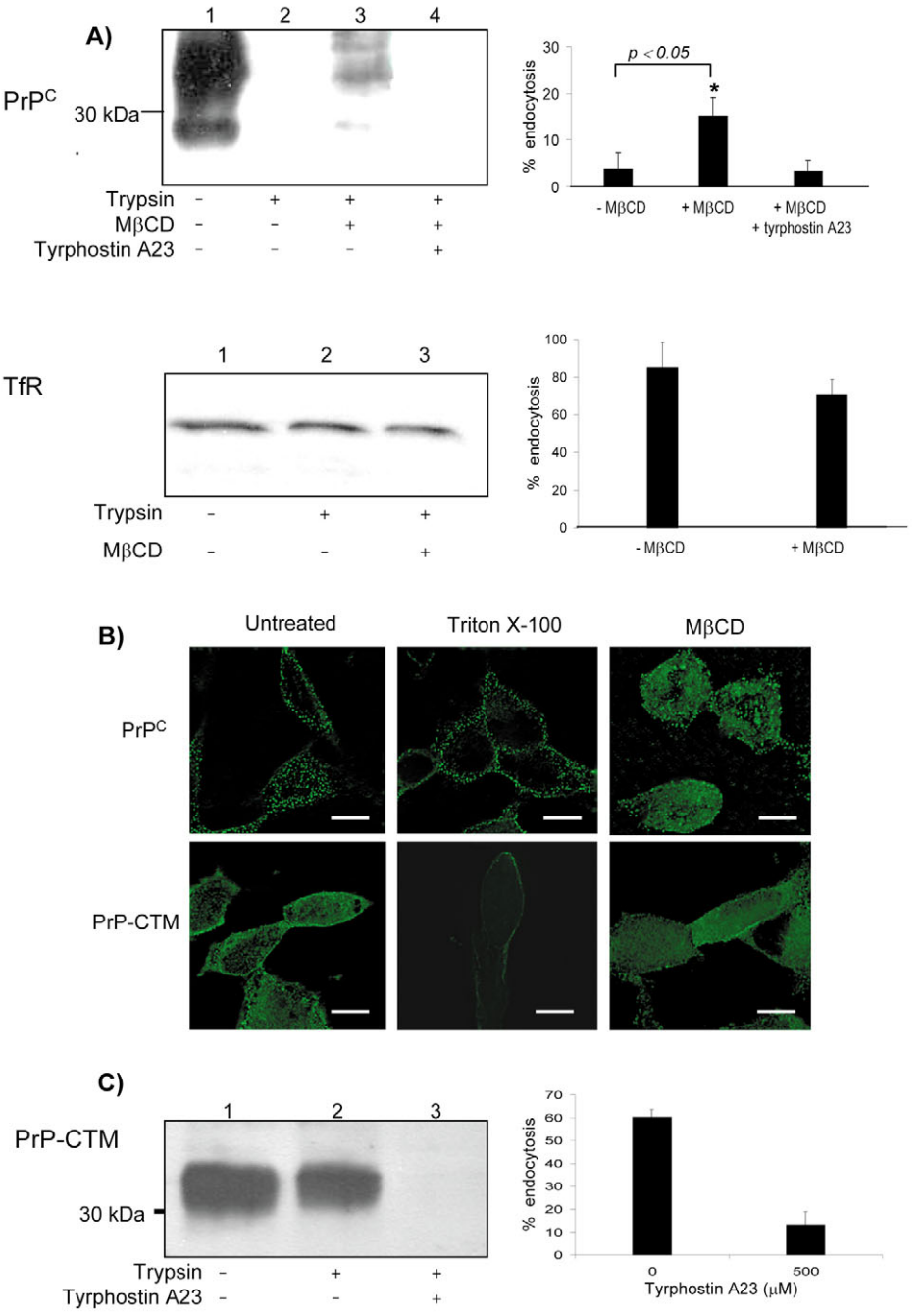

Fig. 7. Disruption of rafts or displacement of PrP from rafts promotes the endocytosis of PrP in the absence of Cu^{2+}. (A) SH-SY5Y cells expressing PrP^C were surface biotinylated and either untreated or treated with 1 mM MβCD in the absence or presence of 500 μM tyrphostin A23. Prior to lysis, the cells were incubated with trypsin to digest cell-surface PrP^C. Cells were then lysed, and PrP^C was immunoprecipitated from the sample using antibody 3F4 and then subjected to western blot analysis. The biotin-labelled PrP^C fraction was detected with peroxidase-conjugated streptavidin. The same samples were also immunoprecipitated using an anti-transferrin receptor antibody. (B) SH-SY5Y cells stably expressing PrP^C or PrP-CTM were seeded onto glass coverslips and grown to 50% confluency. Cells were then pre-incubated with antibody 3F4 at a dilution of 1:1000 in PBS for 30 minutes at 4°C and then washed three times in PBS. Where indicated, cells were incubated either at 4°C for 10 minutes with PBS containing 1% Triton X-100, or with 1 mM MβCD at 37°C for 45 minutes, prior to fixation. Bar, 10 μm. (C) SH-SY5Y cells expressing PrP-CTM were surface biotinylated and either untreated or treated with 500 μM tyrphostin A23. Prior to lysis, the cells were incubated with trypsin to digest cell-surface PrP. Cells were lysed, and PrP-CTM immunoprecipitated and analysed as described in (A). Results are the mean±s.e.m. of three separate experiments done in duplicate each time (n=6), with values of P<0.05 taken as statistically significant where indicated.

cholera toxin in neurons (Shogomori and Futerman, 2001). In the first model, cholera toxin moves out of the detergent-insoluble rafts into clathrin-coated pits. In the second model, the whole raft domain is internalised en bloc by a clathrin-dependent mechanism. By examining the detergent solubility of PrP^C following exposure to Cu^{2+}, we have been able to distinguish between these two models. The observation that PrP^C becomes detergent soluble prior to endocytosis clearly fits with the first model, but not the second, in which the protein would be expected to remain detergent insoluble during endocytosis. Our observation that PrP^C moves out of rafts in the process of being endocytosed is consistent with the observation of Sunyach et al., who used surface biotinylation and sucrose density gradient fractionation, along with electron microscopy colocalisation of PrP^C with the transferrin receptor (Sunyach et al., 2003). Indeed, the ability of PrP^C to move laterally out of lipid rafts upon exposure of the cells to Cu^{2+} is consistent with the model of raft structure proposed by Madore et al. (Madore et al., 1999). On the basis of its differential insolubility in non-ionic detergents, PrP^C was proposed to occupy a position on the outer edges of rafts in a semi-ordered lipid domain from which it would more readily be able to move into the surrounding detergent-soluble regions of the membrane than if it was in the centre of the raft.

What is the mechanism by which Cu^{2+} causes PrP^C to exit detergent-insoluble rafts and move laterally to detergent-soluble regions of the plasma membrane prior to clathrin-mediated endocytosis? Cu^{2+} binding to PrP^C might depress the affinity of the protein for other raft components or Cu^{2+} might promote the interaction of PrP^C with non-raft proteins that can either directly or indirectly associate with clathrin on the cytosolic face of the plasma membrane, thus facilitating internalisation. The latter possibility was suggested by Pauly and Harris (Pauly and Harris, 1998). However, our observations using the octapeptide deletion mutant and a transmembrane anchored form of PrP argue for the former possibility. As shown by immunofluorescence microscopy and buoyant sucrose density gradient centrifugation in the presence of detergent: (1) PrP-Δoct does not leave the rafts on incubation of the cells with Cu^{2+}; (2) disruption of rafts with MβCD promotes the endocytosis of PrP^C; and (3) PrP-CTM, which is not localised in rafts in the plasma membrane, constitutively endocytoses in the absence of Cu^{2+}. This latter observation in particular would not be expected if Cu^{2+} was required to promote the interaction of PrP^C with a protein that engages the clathrin endocytic machinery. It should be noted that the transmembrane and cytosolic domains from angiotensin-converting enzyme used in the PrP-CTM construct (Walmsley et al., 2001) lack recognisable endocytosis signals and that angiotensin-converting enzyme itself does not undergo constitutive endocytosis from the cell surface in the time scale measured here (Warner et al., 2005). Together, these data indicate that binding of Cu^{2+} to the octapeptide repeats is required to promote the dissociation of PrP^C from rafts (summarised in Fig. 8).

Binding of Cu^{2+} to the octapeptide repeats in PrP^C is known to cause a conformational change in the protein (Stockel et al., 1998; Zahn, 2003) and, recently, increasing the concentration of Cu^{2+} has been shown to cause a transition from His-Cu-His intermolecular interactions to Cu-His intramolecular interactions within the octapeptide repeats (Morante et al., 2004). Thus, one possibility is that PrP^C is maintained in rafts through interaction between low levels of Cu^{2+} bound to the octapeptide repeats and another raft-resident molecule. Upon increasing the Cu^{2+} concentration, this intermolecular interaction is disrupted and the octapeptide repeats become fully saturated with Cu^{2+}. Alternatively, another region outside of the octapeptide repeats could interact with a raft-resident molecule and, on binding of Cu^{2+} to the octapeptide repeats, a conformational change in PrP^C disrupts this interaction. This second alternative is supported by the observations that PrP-Δoct, which lacks the octapeptide repeats, is localised in rafts and that a raft-targeting determinant is present in the N-terminal region (residues 23-90) of PrP^C (Walmsley et al., 2003). This N-terminal region of PrP^C was sufficient to mediate raft association when fused to the ectodomain of a non-raft protein (Walmsley et al., 2003). The 23-90 region of the PrP^C ectodomain might function as a raft-targeting determinant either by association with another raft protein or by directly interacting with raft-associated lipids. Although PrP^C has been

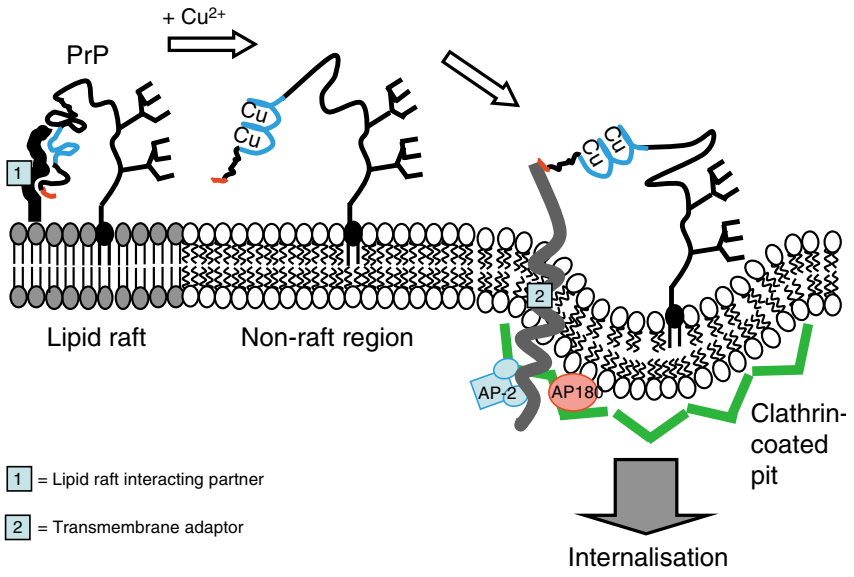

Fig. 8. Schematic showing the mechanism of internalisation of PrP^C. PrP^C is attached to the exoplasmic leaflet of the plasma membrane by its GPI anchor and localises within detergent-insoluble rafts through interactions between its N-terminal region [residues 23-90, red (Walmsley et al., 2003)] and a raft-resident protein or lipid. Upon Cu^{2+} binding to the octapeptide repeats (blue), the protein undergoes a conformational change that dissociates it from the raft-resident partner and PrP^C then moves laterally out of rafts into detergent-soluble regions of the plasma membrane. The polybasic N-terminal region then interacts with the ectodomain of a transmembrane protein that engages, via its cytoplasmic domain, with the adaptor protein AP-2 and the endocytic machinery of clathrin-coated pits.

reported to bind to several proteins, including neuronal cell adhesion molecules [NCAMs (Schmitt-Ulms et al., 2001)], plasminogen (Fischer et al., 2000), the 37 kDa/67 kDa laminin receptor (Hundt et al., 2001) and stress-inducible protein 1 (Zanata et al., 2002), binding in all cases involves regions C-terminal to residue 90. Recently, it has been reported that PrP^C is involved in both cis and trans interactions with NCAM at the neuronal cell surface and that these interactions promote the recruitment of NCAM to lipid rafts (Santuccione et al., 2005). An interaction of PrP^C with raft lipids is supported by the report that PrP lacking a GPI anchor can bind to sphingolipid-cholesterol-rich raft-like liposomes and that this binding is markedly reduced by deletion of the 34-94 region of the protein (Baron and Caughey, 2003). The interaction between the N-terminal region of PrP^C and a raft resident molecule may play a critical role during the process of prion infection. In support of this, the conversion of PrP^C-like proteinase K-sensitive PrP (PrP-sen) to PrP^{Sc}-like proteinase K-resistant PrP (PrP-res) by exogenous PrP-res required a GPI-independent, rather than a GPI-directed, interaction of PrP-sen with sphingolipid-cholesterol-rich raft-like liposomes (Baron and Caughey, 2003).

Although other studies have clearly shown the importance of the N-terminal region of PrP in its endocytosis (Pauly and Harris, 1998; Perera and Hooper, 2001; Shyng et al., 1995), using different mutants of PrP we have shown that the region of the protein that is responsible for dissociating PrP^C from rafts (the octapeptide repeats) is distinct from the region within the protein required for endocytosis (the N-terminal polybasic region). Deletion of just four residues from the N-terminal polybasic region in PrP-ΔN did not prevent the protein moving laterally out of rafts on exposure of the cells to Cu^{2+} but did prevent its endocytosis. Deletions of large regions of the unstructured N-terminus of PrP^C [residues 27-89 (Kiachopoulos et al., 2004) and residues 23-90, 48-93 and 23-51 (Nunziante et al., 2003)] have been reported to disrupt the internalisation of PrP^C, and point mutations within the N-terminal polybasic region disrupted the constitutive endocytosis of PrP^C (Sunyach et al., 2003). However, none of these studies discriminated between the function of the polybasic region and that of the octapeptide repeats. As tyrphostin A23 disrupts the interaction between tyrosine motifs in the cytosolic domains of integral membrane proteins and the adaptor complex AP2 (Banbury et al., 2003), and PrP^C lacks a cytoplasmic domain (as it is GPI anchored), these data provide evidence that the internalisation of PrP^C requires an integral transmembrane protein (Fig. 8). The N-terminal polybasic residues of PrP^C bind to the extracellular domain of this putative transmembrane protein, whereas a YXXΦ motif in its cytosolic domain binds to clathrin-adaptor complexes inside the cell. In addition to this crucial role in the clathrin-mediated endocytosis of PrP^C, the N-terminal polybasic region has been implicated in dynein-mediated retrograde axonal transport (Hachiya et al., 2004), in nuclear targeting of truncated forms of PrP (Gu et al., 2003), in cellular resistance to oxidative stress (Zeng et al., 2003a), in neuroprotective activities of PrP^C (Atarashi et al., 2003; Drisaldi et al., 2004) and in contributing to the action of dominant-negative PrP^C alleles that inhibit conversion of PrP^C to PrP^{Sc} (Zulianello et al., 2000). The identity of the putative transmembrane protein and its role in these other processes await determination.

In conclusion, we have shown that Cu^{2+} promotes the internalisation of PrP^C on neuronal cells through a clathrin-mediated mechanism. Furthermore, we have shown that binding of Cu^{2+} to the octapeptide repeats is required to promote the dissociation of PrP^C from detergent-insoluble lipid rafts and cause it to translocate to detergent-soluble regions of the plasma membrane, whereas the N-terminal polybasic region is required to interact with a transmembrane adaptor protein that couples to the clathrin endocytic machinery.

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