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Fluorimetric Analysis of Copper Transport Mechanisms in the B104 Neuroblastoma Cell Model: A Contribution from Cellular Prion Protein to Copper Supplying

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Abstract Dysregulated body copper homeostasis can negatively impact neuronal functions, but full knowledge of the mechanisms underlying the cell metal distribution has not been achieved yet. The high-affinity copper transporter 1 (Ctr1) is considered the main route for cell copper entry, while the cellular prion protein (PrP^C) is presumed to be involved in the same process. Anchored to the outer side of the plasma membrane, this protein has the ability to bind copper ions and undergo internalization. To provide indications about the contribution of Ctr1 and PrP^C proteins in cell copper transport, we used a fluorimetric method to characterize the kinetic properties of ion internalization in a neuroblastoma cell model, overexpressing prion protein (B104). Biochemical characteristics of intake delineated in the presence of other metal ions and an excess of extracellular potassium were compatible with PrP^C-mediated endocytotic transport. Accordingly, inhibition of clathrin-dependent endocytosis by hypertonic shock and enzymatic removal of surface prion protein reduced copper influx by the same extent. On the whole, experimental evidence collected in a neuron-like cell model sustains a role for PrP^C in mediating copper uptake by clathrin-dependent endocytosis.

Keywords Copper transport · Neuroblastoma cell model · Fluorimetry · Prion protein · Endocytosis

Copper (Cu) is a micronutrient present in the structure of many proteins and enzymes (Pena et al. 1999; Linder and Hazegh-Azam 1996). Although essential to all living organisms, free intracellular ion is virtually nonexistent, as it is able to catalyze the generation of highly reactive oxygen species (Halliwell and Gutteridge 1984, 1990). Levels of free Cu ions have been estimated to be of the order of 10^{-18}–10^{-13} M in yeast cells and in human blood plasma, respectively (Rae et al. 1999; Linder 2001). However, the speed with which ions are taken up and distributed to various compartments (Golgi apparatus, mitochondria, cytosolic proteins) supports the existence of a labile cytosolic pool (Yang et al. 2005). Unfortunately, current knowledge about subcellular Cu compartmentalization and transport kinetics is incomplete and requires advanced technology approaches (i.e., synchrotron X-ray fluorescence microscopy [SXRF], electron microprobe [EM], microparticle X-ray-induced emission [μ-PIXE]), which are not largely available (Ralle and Lutsenko 2009).

Spectrofluorimetric techniques offer a valuable alternative, even though Cu-specific fluorescent sensors compatible with living cells (Coppersensor-1; CTAP-1 [Yang et al. 2005; Zeng et al. 2006]) are not commercially available, unlike the fluorescent transition-metal indicator Phen Green SK. This molecule is a phenantroline covalently attached to fluorescein and, although partly selective toward both Cu and Fe ions, it is a convenient tool to measure cell Cu transport activities, if used under controlled buffer conditions outside. It allowed for assessment of the levels of chelatable iron in isolated rat hepatocytes and liver endothelial cells and analysis of iron fluxes

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across the chloroplast inner envelope membranes (Petrat et al. 1999, 2001; Shingles et al. 2001). The probe was also used to analyze mechanisms of Cu transport into purified lobster hepatopancreatic mitochondria and cells (Chavez-Crooker et al. 2001, 2002) but never in mammal-derived cells.

In this work, we describe the application of Phen Green SK in characterizing kinetic parameters of cell Cu transport in a neuroblastoma-derived cell model. Mechanisms of ion captation were investigated to clarify what modalities are used by cells to internalize Cu, paying attention to the involvement of two major candidates: Ctr1 (Cu transporter 1) and PrP^C (cellular prion protein) proteins. Ctr1 is an integral membrane protein that imports Cu^+ with a high substrate affinity (Nose et al. 2006a; Lee et al. 2000; Zhou and Gitschier 1997), and it is thought to require homotrimerization to form a pore allowing passage of Cu ions through the lipid bilayer (Nose et al. 2006a; Aller and Unger 2006). Evidence from separate groups suggested a role in Cu uptake also for the cellular prion protein PrP^C (Pauly and Harris 1998; Perera and Hooper 2001). PrP^C is a glycoprotein linked to the outer cell membrane by a glycosylphosphatidyl inositol anchor (Kretzschmar et al. 1986; Stahl et al. 1987). Expressed predominantly by neurons at synapses and gliocytes (Salès et al. 1998; Brown et al. 1998), it has been proposed as a mediator of Cu transport (Brown 1999) because of its ability to bind with high-affinity Cu^{2+} ions through its N-terminal-entailed octapeptide domains and to undergo endocytosis (Pauly and Harris 1998; Perera and Hooper 2001; Kramer et al. 2001; Burns et al. 2002; Stockel et al. 1998). Proper Phen Green SK-based transport assays were performed to show whether cell Cu transport occurs through PrP^C-dependent endocytosis phenomena.

Materials and Methods

Cell Culture

B104 neuroblastoma cells derived from rat central nervous system (Schubert et al. 1974) were grown at 37°C, 5% CO_2, in Dulbecco's modified Eagle's medium (Euroclone; Life Sciences Division, Milan, Italy), 1 g/l glucose, supplemented with 10% fetal bovine serum (FBS), 1 mM Na-pyruvate, 2 mM glutamine, and antibiotics (penicillin:streptomycin, 5000 U/ml:5 mg/ml; Sigma-Aldrich, Milan, Italy).

Transport Measurements

The kinetics of Cu intake were characterized under steady-state conditions by a fluorimetric method. To measure ion influx across the plasma membrane of B104 cells, they were first trypsinized and then dye-loaded by incubation for 10 min at 37°C in HBSS (mM: 140 NaCl, 5 KCl, 1 Na_2HPO_4, 1 $CaCl_2$, 0.5 $MgCl_2$, 5 D-glucose, 10 Hepes; pH 7.4) containing 1 μM Phen Green SK (PG SK) diacetate ($\lambda_{exc} = 506$ nm, $\lambda_{em} = 530$ nm; Molecular Probes, Invitrogen, San Giuliano Milanese, MI, Italy). The probe concentration was kept as low as possible, to reduce artifacts from overloading, such as incomplete hydrolysis, compartmentalization, and toxic effects of secondary hydrolysis products. After loading, the cell pellet was washed twice and then resuspended in the physiological buffer cited above. For each evaluation, an aliquot of cells (3×10^4) was introduced in a cuvette housed in a LS-50B Perkin–Elmer spectrofluorometer whose sample compartment had been prewarmed at 37°C. Once the emission signal of the "entrapped" probe was stable, copper chloride was added at different concentrations (0–20 μM) and the rate of fluorescence quenching was monitored (ΔF, 10^{-1} a.u. μg^{-1} protein s^{-1}) as an indicator of Cu influx rate into cytoplasm. During setup procedures, the Cu-chelating agent triethylenetetramine dihydrochloride (Trientine, Teta; Sigma-Aldrich, Milan, Italy) was used in a cuvette at a 20 μM final concentration, to sequester Cu ions outside cells (Rossi et al. 2001) and modify the gradient toward the cytosol.

Effect of Metal Ions and Extracellular Potassium on Cu Transport

To evaluate the effect of selected metal ions (Ag, Cd, Zn, Mn) on cell Cu uptake, B104 cells were first dye-loaded by a 10-min incubation in HBSS containing 1 μM PG SK. Then, during transport measurements 50-fold molar excesses of salt solutions ($AgNO_3$, $CdSO_4$, $ZnCl_2$, and $MnCl_2$) were added to the uptake medium simultaneously with 0.8 μM $CuCl_2$.

To verify the effect of potassium, cells were suspended in HBSS with increasing K^+ concentrations (mM): 5 (control), 75, 110, and 145. Solution osmolarity was maintained by proportional reduction of the NaCl content. A control buffer (5 mM KCl) was used with NaCl totally replaced by choline chloride.

For each condition Cu uptake was calculated as the maximum quenching of fluorescence, normalized by fluorescence emission, before adding copper chloride into the cuvette ($\Delta F_{max}/F_0$), expressed as a percentage of the control.

The overall procedure of biochemical characterization was adapted from Lee et al. (2002).

Dependence of Cu Transport on Internalization Processes

To investigate whether endocytosis processes were involved in cell Cu intake, cells were submitted to

hypertonic shock (600 mM mannitol, 20 min at 37°C) to disrupt the internalization machinery (Hansen et al. 1993; Audet et al. 2005) and then recovered in an iso-osmolar buffer. Maintenance of cell integrity after such treatment was ascertained by Trypan Blue exclusion assay.

Enzymatic Removal of Surface PrP^C by PI-PLC Treatment

Cultures were washed twice with phosphate-buffered salt solution and then preincubated in a serum-free medium containing 5 mU/cm^2 phosphatidylinositol-specific phospholipase C (PI-PLC) from *B. cereus* (Sigma-Aldrich, Milan, Italy) for 2.5 h at 37°C.

Laser-Scanning Confocal Microscopy

B104 cells were plated on four-well chambered coverslips (Sigma-Aldrich, Milan, Italy) coated with either polylysine (Sigma–Aldrich, Milan, Italy; 125 μg/ml) and laminin (BD Biosciences, Milan, Italy; 5 μg/cm^2). Cells were loaded with 0.4 μM PG SK for 2 min at 37°C and then washed twice to remove the excess of the external probe**.** Cultures were exposed to 20 μM $CuCl_2$ with the aim of ascertaining the specificity of the fluorescent emission signal detected by spectrofluorimetric method. The green fluorescence of intracellular PG SK was recorded by using a Nikon TE300 fluorescence microscope equipped with a Nikon C1 Confocal System (excitation source was argon-488). To estimate the ion cellular intake, the intracellular green signal intensity was quantitatively analyzed by ImageJ software, and expressed as density arbitrary units. The temperature on the microscope stage was maintained at 37°C.

Data Analysis

Results for cell culture studies are presented as mean ± SE and each experiment was conducted at least three times. Statistical comparisons were made by Student's *t*-test and ANOVA followed by Dunnett's post test. Significance was demonstrated at $P < 0.05$.

Results

Uptake of Cu Ions in B104 Cultured Cells

The transport kinetics of Cu have been investigated in detail in B104 cells under steady-state conditions. We employed a fluorimetric method based on the Cu-sensitive probe Phen Green SK, whose principle is clearly illustrated in Fig. 1. The exposure of dye-loaded cells (3×10^4) to copper chloride solutions evokes partial quenching of the intensity emission of the entrapped probe, caused by the interaction with Cu ions entering the cells. The entity as well as the speed of this reduction is higher as the extracellular Cu concentration increases (Fig. 1). This encourages the use of Phen Green SK for quantitative purposes. Rapid recovery of the fluorescence emissions can be triggered by the addition of the Cu-chelating agent trientine into the cuvette (Fig. 1). This behavior can be explained by the induced inversion of Cu gradient across the plasma membrane, inducing the cellular efflux of Cu ions which detach from the cytosolic probe.

Concentration Dependence

Cu transport activity in B104 cell model was estimated from the initial rate of fluorescence quenching upon the addition of Cu solutions. The Michaelis–Menten rate constant (K_m) and the maximal velocity (V_{max}) values were determined. The Cu uptake curve depicted in Fig. 2 was fitted to a hyperbole ($r^2 = 0.99$) with respect to a concentration range from 0 to 20.0 μM, this being proof of the expression of a noncooperative and saturable transport system, with a K_m value of 0.25 ± 0.02 μM and a V_{max} of $0.60 \pm 0.01 \times 10^{-1}$ a.u. μg^{-1} protein s^{-1}.

Kinetic data from Fig. 2 were then inspected in an Eadie-Hofstee plot, in which points uniformly converge on a straight line, suggesting the involvement of a single transport system (Fig. 3a). This conclusion is further confirmed by the Hill coefficient value (1.10 ± 0.09), calculated by linear regression on rearranged kinetic data (Fig. 3b).

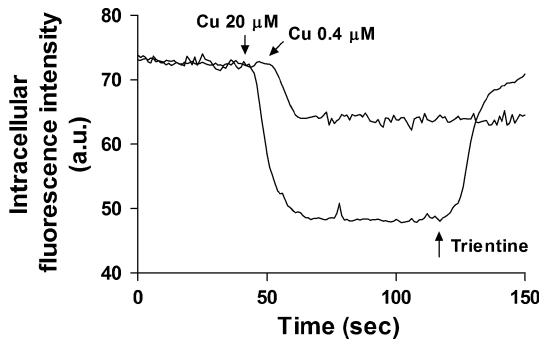

Fig. 1 Typical fluorescence traces obtained from PG SK-loaded B104 cells. Aliquots of dye-loaded B104 cells (see Materials and Methods) were mixed with 0.4 and 20 μM $CuCl_2$ in the external medium. The fluorescence quenching of the entrapped probe is greater and faster for the higher concentration tested. The trace referring to 20 μM Cu-exposed cells shows how the addition of 20 μM trientine induces a dequenching of the fluorescent signal, due to chelation of extracellular Cu ions. Samples were excited at 506 nm and emission was recorded at 530 nm

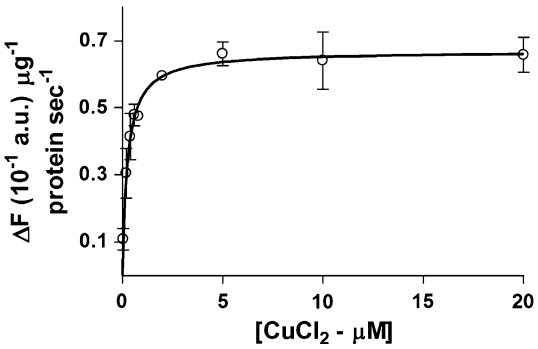

Fig. 2 Rates of Cu uptake as a function of external Cu concentration in PG SK-loaded B104 cells. The best-fit curve is a hyperbolic plot traced by GraphPad Prism version 5.00 (San Diego, CA, USA), suggesting the existence of a saturable Cu transport (K_m, 0.25 ± 0.02 µM). Values are means ± SE of at least three separate recordings for each Cu concentration

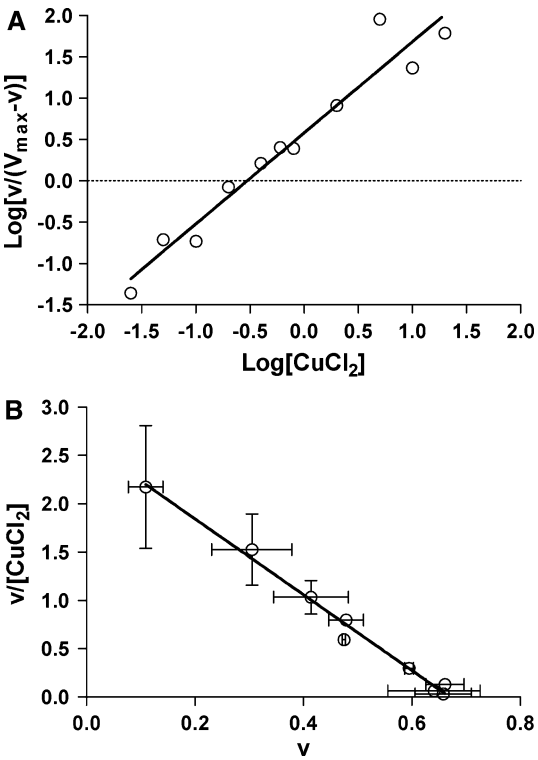

Fig. 3 Mathematical representation of kinetic data from Fig. 2. **a** Hill plot. The average Hill coefficient was estimated from the linear fit of data and its value—1.10 ± 0.09—is suggestive of a single Cu transport system. **b** Eadie-Hofstee plot. The single linear relationship between *v* (velocity) and *V*/[Cu] confirms that the intake process is mediated by a single protein. Points in **a** and **b** are averaged from at least three separate recordings for each Cu concentration

Confocal Microscopy

To make sure that the fluorescence changes measured in the presence of $CuCl_2$ were only attributable to the specific quenching of the intracellular probe, preloaded adherent cells were imaged by confocal microscopy to localize the source of the signal. As shown in Fig. 4, the PG SK probe is diffusely detectable inside the cells, and upon the addition of 20 µM copper chloride its fluorescent emission undergoes rapid quenching (36.0 ± 4.5%), within 1 min ($P < 0.01$). The emission intensity remains stable during the following 2 min, to reach 45.0 ± 3.1% by the end of recording (5 min). The sequence of sectional images in Fig. 4 confirms the specificity and reliability of the fluorimetric method.

Biochemical Characterization

Cu transport was further characterized by measuring the entity of ion intake in the presence of Ag, Cd, Mn, and Zn ions applied to the uptake medium simultaneously with Cu at a 50-fold higher concentration than that of the latter. Comparisons were made by testing a unique Cu concentration—0.8 µM—for which the rate of intake is in the ascending region of the uptake curve (Fig. 5).

Silver ions seemed to be ineffective as inhibitors of Cu (Fig. 5). In the case of other metal cations, transport assays evidenced a noticeable reduction of Cu uptake with the exception of manganese. In particular, a pronounced decrease was registered for Zn (81.7% ± 2.3% reduction) and Cd (65.4% ± 0.0% reduction; Fig. 5).

The high availability of potassium has been reported to stimulate Ctr1-mediated Cu transport (Lee et al. 2002), so we wanted to analyze to what measure this factor affects Cu kinetics in the B104 cell model.

To study the dependence of Cu flux on extracellular potassium, fluorimetric assays were conducted on cells resuspended in HBSS containing 5–145 mM potassium chloride. Copper salt solution was added to a final concentration of 0.8 µM. For every condition, the effects on the entity of intake were statistically irrelevant compared to control conditions (Fig. 6), in disagreement with the trend delineated by Lee et al. (2002) for the high-affinity Cu transporter Ctr1.

Effect of Hypertonic Stress and Surface Prion Protein Removal on Cu Intake

Exposure of B104 cells to 600 mM hypertonic mannitol, known to delay clathrin-dependent endocytosis phenomena (Hansen et al. 1993; Audet et al. 2005), determined a remarkable reduction of the absolute Cu intake (Fig. 7). To investigate whether prion protein was the interpreter of such internalization processes, surface molecules were removed by treating cells with a PI-PLC enzyme solution for 2.5 h. Total removal of prion protein from the plasma membrane was demonstrated by Western blotting analysis

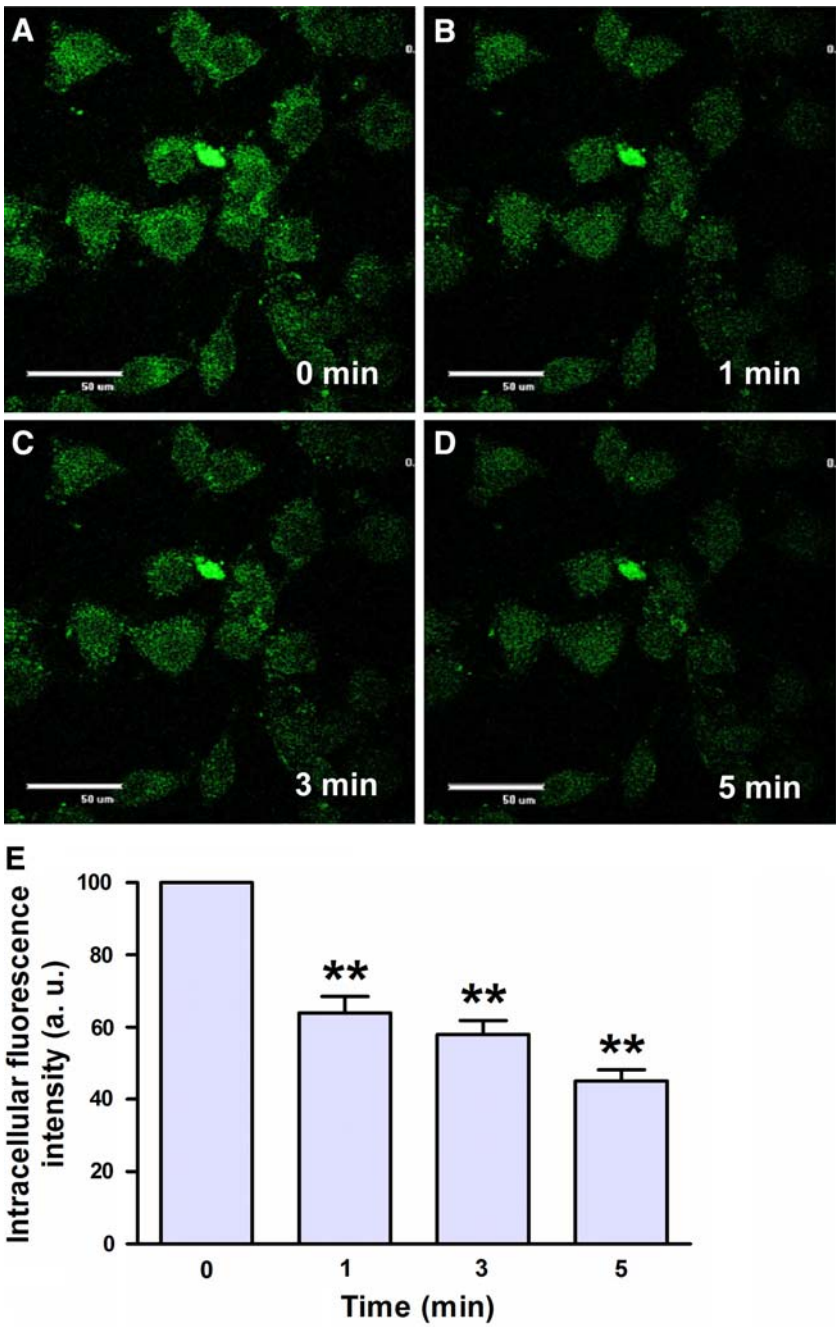

Fig. 4 Effect of intracellular Cu concentration on PG SK probe fluorescence emission. B104 cells were grown on chambered coverslips and dye-loaded as described under Materials and Methods. Subcellular probe emission was recorded using laser-scanning confocal microscopy (scale bar, 50 μM) and images were collected before (0 min; **a**) and after (**b**–**d**) the addition of 20 μM $CuCl_2$. Green levels at each time point proportional to intracellular Cu were quantified by Image J software (**e**). Graphed values (expressed as a percentage of initial fluorescence) are means ± SE of emission intensities of 10–15 cells. Comparable results were obtained from three separate assays. ** $P < 0.01$, ANOVA

(data not shown). The enzymatic treatment was found to reduce the uptake of Cu ions (Fig. 7). To exclude the possibility of other proteins besides PrP^C being involved in mediating ion transport through endocytosis, cells were exposed to the enzyme solution and subjected to hypertonic shock after dye-loading. Both conditions failed to induce any further reduction of Cu transport than that observed under the enzymatic treatment alone (Fig. 7). This indicates that the contribution of prion protein is fundamental and it represents a restricting step in the overall pathway of cell Cu import.

As a control, the Na^+/H^+ exchange activity (not endocytic and PrP^C-independent) was analyzed by a BCECF (2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein)-based fluorimetric method, in parallel to the measurement of Cu uptake (see Supplementary Material). As expected, it was found to be unchanged by the treatments used, thus confirming the specificity of results obtained.

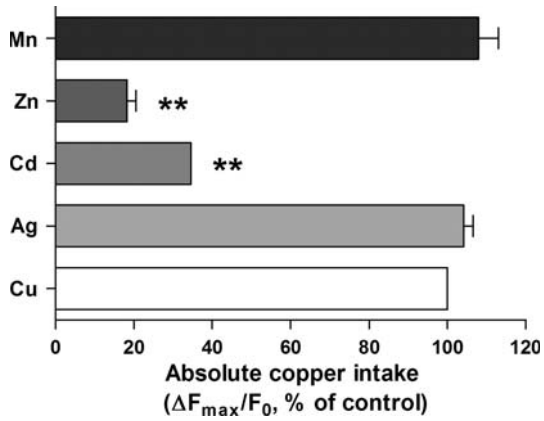

Fig. 5 Effect of silver, zinc, cadmium, and manganese ions on Cu intake in B104 cell model. Aliquots of PG-SK-loaded B104 cells (3×10^4) were simultaneously exposed to 0.8 μM $CuCl_2$ and 50-fold molar excesses of Ag^+, Zn^{2+}, Cd^{2+}, and Mn^{2+} ions. The absolute Cu intake was evaluated as a percentage of the ion accumulation measured in the absence of cited metal ions. Experiments were repeated at least three times. ** $P < 0.01$, ANOVA

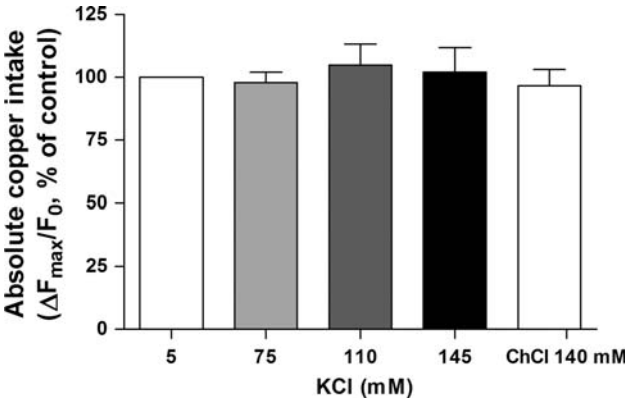

Fig. 6 Effect of K^+ on Cu uptake in PG SK-loaded B104 cells. Cells were suspended in buffers with varying K^+ contents. Aliquots of cell suspensions were exposed to 0.8 μM $CuCl_2$ and ion intake was calculated as a percentage with respect to control conditions. Data are representative of at least three experiments

Discussion

Copper is an essential trace metal, playing a critical role as a cofactor for a number of enzymes (Pena et al. 1999; Linder and Hazegh-Azam 1996). Most of them are linked to the functions of nervous tissue, and consistently, some features of neurodegeneration arise from deregulated Cu homeostasis (Zucconi et al. 2007; Waggoner et al. 1999; Zatta and Frank 2007). Despite the importance of a proper Cu supply, mechanisms actuated by cells to correctly accomplish ion uptake have not been positively identified yet. Ctr1 transporter is currently believed to drive Cu import with a high affinity and specificity (Nose et al. 2006a; Lee et al. 2000; Zhou and Gitschier 1997), but it can be questioned in the light of some experimental evidence

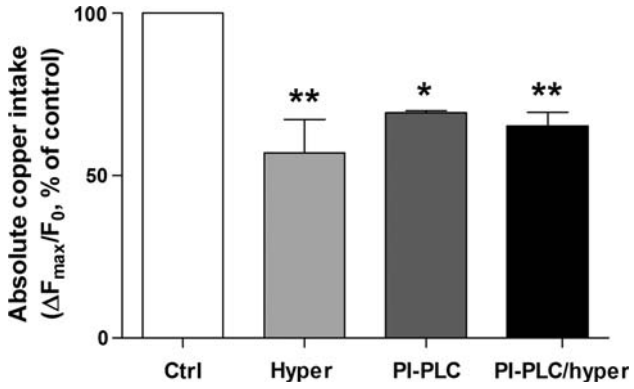

Fig. 7 Dependence of Cu uptake on PrP^C-mediated endocytosis processes. Cu transport activity in the presence of 0.8 μM $CuCl_2$ was evaluated in dye-loaded cells under control conditions (Ctrl), after exposure to hypertonic shock (Hyper), after enzymatic removal of surface prion protein (PI-PLC), and after exposure of PI-PLC-treated cells to hypertonic shock (PI-PLC/hyper). Intake was calculated as a percentage with respect to control conditions. Experiments were performed at least in triplicate. * $P < 0.05$ and ** $P < 0.01$, ANOVA

(Nose et al. 2006b; Klomp et al. 2002). Enterocytes from *Ctr1*$^{-/-}$ mice show a more than eightfold accumulation of Cu compared to the *wild-type* genotype (Nose et al. 2006b). In addition, the hepatic deletion of Ctr1 expression in mice has been shown not to strikingly and irreversibly affect Cu concentrations in other organs, supporting the hypothesis of alternative methods of cell Cu uptake (Kim et al. 2009).

Some properties of cellular prion protein (PrP^C) make it a good alternative means of copper supply with respect to Ctr1 protein. At steady state, most PrP^C is located on specific plasma membrane sites known as rafts (Taylor and Hooper 2006). The addition of micromolar concentrations of Cu^{2+} ions to cells was found to rapidly stimulate endocytosis of prion protein (Pauly and Harris 1998; Perera and Hooper 2001; Brown and Harris 2003; Taylor et al. 2005). This process was established to be dependent on the ability of PrP^C to specifically bind up to four Cu^{2+} ions through the octapeptide repeats (sequence PHGG(G/S)WGQ) within 59–90 residues at the N-terminal half (Stockel et al. 1998; Viles et al. 1999; Hornshaw et al. 1995; Walter et al. 2006). Considering that these sequences show a weak affinity for monovalent Cu or other metal species (Stockel et al. 1998; Whittal et al. 2000; Millhauser 2004) and that binding sites are almost saturated at Cu concentrations close to physiological values in the body (Kramer et al. 2001; Burns et al. 2002; Hartter and Barnea 1988a; Watt and Hooper 2005), PrP^C could probably drive Cu^{2+} delivery across the plasma membrane. As proof, synaptosomes, endosomes (Herms et al. 1999; Brown et al. 1997a; Brown 2003), and whole brain and liver extracts (Brown et al. 1997a) from *Prn-p*$^{-/-}$ mice exhibit a reduced Cu content, by up to 10-fold (Brown et al. 1997a), compared to the wild-type preparations. An increase in prion

protein expression in cerebellar cultures has been related to elevated levels of Cu,Zn superoxide dismutase activity, depending on the enhanced delivery of Cu to the intracellular enzymatic targets (Brown et al. 1997b; Brown and Besinger 1998). Finally, unpublished results from our laboratory demonstrated that B104 neuroblastoma cells, when Cu deprived for increasing time periods, mount a transcriptional response consisting of a striking and scalar increase in PrP^C expression, while Ctr1 transcript levels are only slightly increased and protein amount remains unaffected (Urso E, unpublished data).

Based on these observations, we wanted to investigate the hypothesis that prion protein has a direct role in high-affinity Cu transport activity. We preliminarily characterized Cu kinetics under steady-state conditions in an appropriate cell model—the B104 neuroblastoma—overexpressing prion protein (Monnet et al. 2003). We employed a spectrofluorimetric method based on the use of the fluorescent probe Phen Green SK, never used before in mammal-derived cells to measure Cu fluxes.

In our study, we examined the precocious fluxes of Cu toward the cytosol of B104 neuroblastoma cells, exposing them to 0–20 μM Cu(II), which is a range of values overlapping with those found in the body, from 0.5–2.5 μM in the cerebrospinal fluid (Hartter and Barnea 1988b) to 8 μM, corresponding to the pool of plasma exchangeable ion (Kramer et al. 2001; Burns et al. 2002), up to 10–300 μM at synapses (Hartter and Barnea 1988a; Watt and Hooper 2005).

The uptake curve we obtained is consistent with the expression of a saturable transport system that, at first glance, could be considered compatible with the characteristics of the Ctr1 transporter family (Lee et al. 2002). Nevertheless, substrate affinity (K_m, 0.25 ± 0.02 μM) appears to be higher than expected in that case, where K_m values range from 0.6 μM in C6 rat glioma cells to 11–13 μM in isolated murine hepatocytes (Lee et al. 2002). As deduced from both Hill and Eadie-Hofstee plots fitting a straight line, Cu transport occurs through a single transport system.

Specific assays were performed to verify the Cu transport activity of a good candidate, as we have considered the cellular prion protein PrP^C to be, based on experimental evidence above.

Cells were preliminarily exposed to hypertonicity in order to prevent clathrin-coated pit-mediated endocytosis (Hansen et al. 1993; Audet et al. 2005; Brown and Harris 2003). Treatment with hypertonic mannitol heavily compromised the uptake of Cu, suggesting that internalization phenomena through clathrin-coated pits are a fundamental prerequisite for transport mechanisms. Unfortunately this finding was not sufficient to discriminate between PrP^C and Ctr1 transporter contributions, as low Cu concentrations were previously found to induce the clearance of hCTR1 proteins from the plasma membrane of HEK293 cell line in just 2 min (Petris et al. 2003). The enzymatic removal of PrP^C molecules from the cell surface caused a reduction in Cu flux, this being a proof of the direct involvement of prion protein in cellular Cu trafficking. No further decrease in Cu uptake was observed after exposure of PI-PLC-treated cells to hypertonicity, indicating that the exclusive interpreter of Cu-dependent endocytosis phenomena is the cellular prion protein. The Hill coefficient calculated for Cu concentrations tested (1.10 ± 0.09) is indicative of low-occupancy coordination binding of metal ions to the N-terminus of PrP^C (1:1 stoichiometry [Klewpatinond et al. 2008]), associated with the higher-affinity interaction mode (K_d, 3 nM [Wells et al. 2006]). This observation is further endorsed by SERS (surface enhanced raman spectroscopy) findings on Cu-exposed B104 cells (Manno D, Urso E, unpublished data), showing that interaction of prion protein, in its physiological context, and copper occurs through a multiple-histidine coordination of a single ion [Cu(II)-Nτ_4] even at a high Cu concentration (>20 μM).

We collected a series of experimental data concerning the biochemical characteristics of Cu transport in a B104 cell model. Silver ions, previously shown to inhibit hCtr1-mediated Cu transport in HEK293 cells (Lee et al. 2002), did not affect the cellular uptake of this ion. This finding strongly suggests that Ctr1 proteins may not be the main interpreters of cellular Cu intake. Transport assays were repeated to evaluate Cu transport activities in the presence of other metal ions, that is, Cd, Zn and Mn. This last element did not evoke a significant inhibitory effect, while Cd and Zn ions heavily compromised Cu uptake, as reported before (Lee et al. 2002). The outcomes concerning zinc effects are consistent with the hypothesis that Zn ions compete with Cu for PrP^C-mediated transport: extracellular zinc, analogous to Cu ions, has been demonstrated to cause prion protein to be rapidly internalized from the plasma membrane to endocytic and Golgi compartments in N2a neuroblastoma cells (Brown and Harris 2003). Zinc-dependent endocytosis of prion protein was found to occur in response to ion concentrations detectable in some brain regions (Smith 1983). Cd, Mn, and Zn ions have been hypothesized to displace Cu from the PrP^C protein, so determining a conversion into an intermediate isoform rich in β-sheet uctures (Choi et al. 2006), which could modify the physiological function of protein. Consistently with the involvement of endocytosis in Cu uptake, Cd has been shown to impair the efficiency of that process determining a defect in endosomal acidification (Choi et al. 1999). The lack of transport activity inhibition by manganese may be due to the low affinity toward prion protein at pH 7.5 and to the low concentration we tested, this being the highest binding affinity observed at pH 5.5 (Brazier et al. 2008).

Although not representing a direct demonstration of the involvement of prion protein in the endocytic transport of Cu ions, results just discussed strengthen this finding.

Increasing levels of extracellular K^+ did not induce any significant change in the entity of Cu intake. Since it has been shown in HEK293 cells that high K^+ stimulates hCtr1-mediated Cu transport (Lee et al. 2002), our findings suggest that Ctr1 protein does not have a role in this process in a B104 cell model.

In summary, taken together our data indicate that in the B104 neuron-like cell line, the cellular prion protein drives copper internalization through clathrin-dependent endocytosis. This finding is of great importance, as dysregulation of cell Cu availability deriving from loss of PrP^C function could partly account for the pathophysiology of prion diseases.

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