Induction of cellular prion protein gene expression by copper in neurons

Lorena Varela-Nallar,1,2 Enrique M. Toledo,1 Luis F. Larrondo,2 Ana L. B. Cabral,3,4 Vilma R. Martins,3 and Nibaldo C. Inestrosa1,2

1Centro de Regulación Celular y Patología “Joaquín V. Luco”, and 2Millenium Institute for Fundamental and Applied Biology, Facultad de Ciencias Biológicas, P. Universidad Católica de Chile, Santiago, Chile; 3Ludwig Institute for Cancer Research, São Paulo; and 4Departamento de Bioquímica, Instituto de Química da Universidade de São Paulo, São Paulo, Brazil

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Prion diseases are a group of fatal transmissible neurodegenerative disorders, which includes kuru and Creutzfeldt-Jakob disease in humans, scrapie in sheep and bovine, spongiform encephalopathy in cattle (48). These pathologies are caused by the conformational transition of the native α-helical cellular prion protein (PrPα) into a β-sheet pathogenic isoform. However, the normal physiological function of PrPα remains elusive. We report herein that copper induces PrP expression in primary hippocampal and cortical neurons. PrP induced by copper has a normal glycosylation pattern, is proteinase K-sensitive and resistant to digestion with proteinase K (PK) (13). PrP expression in neurons.

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MATERIALS AND METHODS

Primary culture of rat hippocampal and cortical neurons. Rat hippocampal and cortical cultures were prepared as described previously with some modifications (1, 11). Hippocampi and cortices from Sprague–Dawley rats at embryonic day 18 were removed, dissected free of meninges in Ca²⁺/Mg²⁺-free Hanks’ balanced salt solution (HBSS), and rinsed twice with HBSS by allowing the tissue to settle to the bottom of the tube. After the second wash, the tissue was resuspended in HBSS containing 0.5% (wt/vol) trypsin and incubated for 15 min at 37°C. After three rinses with HBSS, the tissue was mechanically dissociated in plating medium (Dulbecco’s modified Eagle’s medium (GIBCO, Rockville, MD)), supplemented with 10% horse serum (GIBCO), 100 U/ml penicillin, and 100 μg/ml streptomycin by gentle passage through Pasteur pipettes. Dissociated hippocampal and cortical cells were seeded in poly-l-lysine-coated 6-well culture plates at a density of 7 × 10⁵ and 1 × 10⁶ cells per well, respectively, in plating medium. Cultures were maintained at 37°C in 5% CO₂ for 2 h before the plating medium was replaced with neurobasal growth medium (GIBCO) supplemented with B27 (GIBCO), 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. This method resulted in cultures highly enriched for neurons (~5% glia). After 6 days in culture, cells were used for the experiments. Cells were treated with CuCl₂ or a copper-glycine complex that was synthesized with the use of CuCl₂ and a 2 M excess of glycine, and the pH was adjusted to 7.4 (7). BCS was purchased from Sigma (St. Louis, MO). Quantification of the total cellular copper content was performed as previously described (57).

Cell lines culture. Rat PC12 pheochromocytoma and C6 glioma clones stably transfected with a luciferase reporter vector driven by the rat Prnp promoter region (~2,831 to +47 bp) (52) have been previously described (10). PC12 cells were routinely grown in RPMI medium supplemented with 10% FBS (GIBCO), 100 U/ml penicillin, and 100 μg/ml streptomycin. C6 cells were grown in RPMI medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Both cell lines were maintained at 37°C in 5% CO₂ atmosphere. Treatments were done in RPMI medium supplemented with antibiotics.

Reverse transcriptase-polymerase chain reaction. Total RNA from primary hippocampal neurons was prepared using TRIzol (Invitrogen, Rockville, MD) according to manufacturer’s instruction. Total RNA (3 μg) was subjected to reverse transcription in a final volume of 25 μl comprising 50 pmol oligo dT, 0.5 mM dNTPs, 10 mM DTT, 2.5 mM MgCl₂, 2 μl of reaction buffer, and 50 units of Super Script II reverse transcriptase (Invitrogen) for 50 min at 42°C. For PCR, amplification primers used were the following: PrP; sense, 5’-TCATCCCACGATCAGGAAGATGGC-3’; antisense, 5’-TCATCCCACTGATCAAGAAG-3’; actin; sense, 5’-AGAGGGAATCTCGGCTGAC-3’; antisense, 5’-GACTCATCTGATCTCTGGTG-3’. PCR reaction mixture included 2 μl cDNA, 50 pmol of each primer, 0.2 mM dNTPs, 3 mM MgCl₂, 2.5 μl reaction buffer, and 1.25 units of Taq DNA polymerase. Platinum (Invitrogen) in a final volume of 25 μl. Twenty-five cycles of denaturation at 95°C for 0.5 min, annealing at 62°C for 0.5 min, and extension at 72°C for 1 min were performed. Specific PCR products were run on ethidium bromide-stained agarose gel (1.5%) and visualized under UV light.

Cell lysis and PK digestion. Neurons growing on 6-well culture plates were rinsed twice with ice-cold PBS and lysed with 300 μl of ice-cold lysis buffer (10 mM Tris–HCl, pH 7.8, 100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, and 0.5% sodium deoxycholate) on ice for 30 min. Nuclei and large debris were removed by centrifugation at 1,000 g for 5 min (4°C). The proteins in the postnuclear supernatants were precipitated by the addition of 4 volumes methanol (100%) at ~20°C, incubated for at least 30 min, and then collected by centrifugation. For PK digestion, the protease was added to the postnuclear supernatant to a final concentration of 20 μg/ml and proteolysis was performed for 30 min at 37°C. The reaction was stopped by the addition of PMSF at a final concentration of 3 mM and incubating the samples on ice for 5 min. Proteins were precipitated with 4 volumes methanol. Precipitated samples were sonicated in SDS-PAGE sample buffer and one-third of postnuclear supernatant samples and total PK-treated samples were analyzed by immunoblotting.

Phosphatidylinositol-specific phospholipase C treatment. For release of plasma membrane PrP<sup>+</sup> hippocampal neurons were incubated with phosphatidylinositol-specific phospholipase C (PIPLC; 10 μ/ml) (Molecular Probes, Eugene, OR) in serum-free Opti-MEM medium (GIBCO-BRL). After 24 h the media was recovered and proteins were precipitated with 10% TCA for 30 min and then collected by centrifugation. Neurons were rinsed twice with ice-cold PBS and lysed with 300 μl of ice-cold lysis buffer as described above. The proteins in the postnuclear supernatants were precipitated with 10% TCA. Precipitated samples were sonicated in SDS-PAGE sample buffer and 1/4 of postnuclear supernatant and the total protein released to the media were analyzed by immunoblotting.

Immunoblot analysis. Proteins were resolved in SDS-PAGE (14% polyacrylamide), transferred to PVDF membrane and reacted with 8H4 monoclonal antibody (kindly provided by Dr. Man-Sun Sy, Case Western Reserve University School of Medicine), anti-recombinant H<sub>1</sub>-PrP<sup>+</sup> antiserum produced in Prnp<sup>−/−</sup> mouse (M-203), goat polyclonal anti-PrP<sup>+</sup> (M20, Santa Cruz Biotechnology, Santa Cruz, CA), goat polyclonal anti-H-SP70 (Santa Cruz Biotechnology), monoclonal anti-β-actin (Abcam, Cambridge, MA), rabbit polyclonal anti-β-τ-bulin (Santa Cruz Biotechnology). The reactions were followed by incubation with anti-mouse, anti-rabbit or anti-goat IgG peroxidase labeled (Pierce, Rockford, IL) and developed using the ECL technique (Amersham Biosciences, Piscataway, NJ).

Immunofluorescence. Hippocampal neurons were seeded onto poly-l-lysine-coated coverslips in 24-well culture plates at a density of 5 × 10⁴ cells per well. After 6 days in the culture, the cells were used for the experiments. After treatment, the cells were rinsed twice in ice-cold medium and fixed with a freshly prepared solution of 0.4% paraformaldehyde in PBS for 20 min and permeabilized for 10 min with 0.2% Triton X-100 in PBS. After several rinses in ice-cold PBS, the cells were then incubated in 0.2% gelatin in PBS (blocking solution) for 30 min at room temperature, followed by an overnight incubation at 4°C with the monoclonal antibody 8H4 diluted 1:100. The cells were extensively washed with PBS and then incubated with Alexa 555-conjugated anti-mouse IgG (1:2,000) for 1 h at room temperature. The cells were mounted in mounting medium and analyzed in a Zeiss confocal laser microscope.

Plasmid construction and transient transfection. Five fragments of the rat Prnp promoter gene (GenBank accession no. D50092) (52) were amplified using the following sense primers: 5’-TTAAGCTTATCGTATCTGATGCTTG-3’; antisense, 5’-TCATCCCACTGATCAAGAAAGA-3’; actin; sense, 5’-AGAGGGAATCTCGGCTGAC-3’; antisense, 5’-GACTCATCTGATCTCTGGTG-3’. PCR reaction mixture included 2 μl cDNA, 50 pmol of each primer, 0.2 mM dNTPs, 3 mM MgCl₂, 2.5 μl reaction buffer, and 1.25 units of Taq DNA polymerase in a final volume of 25 μl. Twenty-cycle cycles of denaturation at 95°C for 0.5 min, annealing at 62°C for 0.5 min, and extension at 72°C for 1 min were performed. Specific PCR products were run on ethidium bromide-stained agarose gel (1.5%) and visualized under UV light.

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**Luciferase assay.** Stably and transiently transfected cells exposed to treatments were rinsed three times in PBS and harvested in 100 µl of 1× luciferase cell culture lysis reagent (Promega). The luciferase activity in cell lysates was measured as relative light units by using the Promega Luciferase Assay System according to the manufacturer’s protocol, with a MD3000 Luminometer (Analytical Luminescence Laboratory). Relative luciferase units of stable clones were normalized for protein concentration, and normalized luciferase activity of each stable clone after treatment was expressed as the fold increase over the equivalent clone without treatment. For transient transfection, relative luciferase units were normalized for β-gal activity, and normalized luciferase activity of cells subjected to treatments were expressed as the fold increase over the untreated cells transfected with the same plasmid.

Electrophoretic mobility shift assay. The MLS1 probe used was the following: sense, 5 ’-CAGCTCTCCCTCCGCTCCCCTGGCCATCTCTG-3’; antisense, 5 ’-TGTCAGAGATGGCAGGGGACGCAGGAGGAGTGGGTA-3’. The probe was end labeled using T4 polynucleotide kinase and [γ-32P]ATP. PC12 nuclear extracts were prepared as previously described (2). EMSAs were performed with the Promega GelShift assay system (Promega) according to the manufacturer’s protocol, using 3.5 µg of nuclear extract and 0.5–2 × 10^5 cpm of the radiolabeled DNA probe per sample. For supershift experiments, 1 µl of anti-MTF-1 antibody (generously provided by Dr. Glen Andrews, Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, KS) was added to the mixture previously to the addition of the labeled DNA probe.

**Statistical analysis.** Each experiment was performed in triplicate and mean values represent at least three independent experiments. Statistical significance was tested by Student’s t-test. P < 0.05 was considered statistically significant.

**RESULTS**

Copper induces the expression of PrP<sup>C</sup> in cultured rat neurons. Considering that PrP<sup>C</sup> is normally expressed in neuronal tissues, the effect of copper on the expression of Prnp was assessed in cultured neurons. Total RNA isolated from primary cultured hippocampal neurons with or without treatment with 50 µM CuCl<sub>2</sub> for 12 h was analyzed by RT-PCR to evaluate the expression level of Prnp. Densitometric analysis normalized to β-actin mRNA revealed an increase in Prnp mRNA level after copper treatment (Fig. 1A). To evaluate copper modulation at the protein level, hippocampal and cortical neurons were treated with different concentrations of CuCl<sub>2</sub> for 24 h and then harvested for immunoblotting analysis of PrP<sup>C</sup> using the monoclonal antibody 8H4 (68) and β-tubulin as a loading control. Three distinct bands were observed in hippocampal and cortical neurons (Fig. 1, A and B, C, see arrowheads). The graphs show densitometric analysis of PrP<sup>C</sup> after normalization to β-tubulin. As observed, PrP<sup>C</sup> level was significantly induced by 50 µM CuCl<sub>2</sub> in both hippocampal and cortical neurons. Similar induction was observed when membranes were probed with anti-rPrP<sup>C</sup> antiseraum produced in Prnp<sup>0/0</sup> mouse, or with a goat polyclonal anti-PrP<sup>C</sup> antibody (not shown).

To mimic the in vivo situation where the exchange of a metal ion is from one protein or ligand to another, copper was also supplied as a copper-glycine complex (7). Because glycine competes for free copper, nonspecific binding to low-affinity

**Fig. 1.** Copper induces the expression of cellular prion protein (PrP<sup>C</sup>) in primary cultured neurons. A: RT-PCR analysis of the expression of PrP<sup>C</sup> in primary hippocampal neurons untreated or treated with 50 µM CuCl<sub>2</sub> for 12 h. Densitometric analysis of visualized bands was carried out with NIH Image J software (version 1.33), and PrP was normalized against β-actin mRNA levels and expressed as the fold increase compared with control cells. Western blot analysis of total cell extracts from hippocampal (B) or cortical (C) neurons untreated or treated with 25, 50, and 100 µM CuCl<sub>2</sub> for 24 h, with anti-PrP monoclonal antibody 8H4 or anti-β-tubulin polyclonal antibody. Arrowheads indicate the 3 distinct bands observed. Molecular size markers are indicated as bars on the left side of the panels and represent 36, 24, and 19 kDa. PrP was normalized to β-tubulin levels and expressed as relative (fold) increase over untreated neurons. Bars are means ± SE of 3 independent experiments. *P < 0.05 vs. the single mean Student’s t-test.
sites is avoided. Like CuCl₂, treatment with 50 µM copper-glycine complex for 24 h induces PrPSc levels in hippocampal neurons (Fig. 2B). The induction of PrPSc levels by CuCl₂ and copper-glycine complex was inhibited by cotreatment with the Cu²⁺-specific chelator bathocuproinedisulfonic acid (BCS) (Fig. 2, A and B). This result indicates that elevation of intracellular copper concentration causes the increased expression of PrPSc. We determined that treatment with 50 µM CuCl₂ for 24 h increased total cellular copper content 5.36 ± 0.32-fold in hippocampal neurons.

The toxicity of copper treatment was assessed by MTT assay. Cell viability of hippocampal neurons was 91.7 ± 10.6% of the control cells after incubation with 50 µM CuCl₂ for 24 h and 95.3 ± 4.4% after treatment with 50 µM copper-glycine complex. The LC₅₀, the concentration that killed half of the cells, was calculated to be 161.9 µM CuCl₂ after 24 h of treatment. To determine whether copper treatment induced a stress response that could mediate the effect on the expression of PrPSc, we also analyze the effect of copper on the expression of the 70-kDa heat shock protein (HSP70). HSP70 is stress protein induced in response to different stimuli, including heat shock and oxidative stress (26, 37, 39), and therefore has been proposed as a general marker of cellular damage. Treatment with 50 µM CuCl₂ or copper-glycine complex for 24 h did not induce HSP70 levels (Fig. 2, A and B), suggesting that these treatments did not induce a general stress response. This is in agreement with a previous report showing that copper concentration required for the induction of HSP70 expression is >200 µM (42).

To assess whether the three bands observed correspond to the β-, mono-, and unglycosylated forms of PrPSc, hippocampal neurons were treated with 5 µg/ml tunicamycin in the presence or absence of 50 µM CuCl₂ for 24 h. In both, control and copper treated neurons, there was an enrichment of the lower molecular weight band compared with the other bands, indicating it corresponds to the unglycosylated form of PrPSc (Fig. 3A). As expected, an increase in the unglycosylated form of PrPSc was observed in copper treatment in the presence of tunicamycin. Apparently no changes in the PrPSc glycosylation pattern were induced by copper (Fig. 1B and 3A, in the absence of tunicamycin).

In vitro, copper converts PrPc into a detergent-insoluble and PK-resistant specie (49, 66) suggesting that copper may have a role in the conversion of PrPSc into PrPSc. To determine whether copper induces PK resistance of PrPSc in neurons, detergent extracts of hippocampal neurons incubated with different concentrations of CuCl₂ for 24 h were digested with PK and then assayed by immunoblotting with anti-PrP 8H4. As shown in Fig. 3B, no PK-resistant PrP was detected in control or copper-treated neurons.

It has been observed in N2a cells and hippocampal neurons that copper induces detergent insoluble PrP, suggesting it induces PrPSc misfolding and aggregation (28, 62). This aggregation may be associated to the biosynthetic pathway and occurs during normal protein folding or intracellular trafficking. To study whether PrPSc reaches the cell surface in the presence of copper, neurons were treated with PIPLC during copper exposure. As shown in Fig. 3C, there was a decrease of PrPSc in the cell extracts from hippocampal neurons treated with 10 nM/ml PIPLC in the presence or absence of 50 µM CuCl₂ for 24 h, indicating that most of PrPSc got to the cell surface during the treatment. In agreement, there was an increase of PrPSc in the media of PIPLC-treated neurons (Fig. 3D). PrPSc detected in the media migrates as the mature biglycosylated form of PrPSc. The increase of PrPSc in the medium of PIPLC-treated neurons was higher in the presence of copper, supporting that the metal induces the expression of cell surface PrPSc.

The effect of copper on PrPSc level was also evaluated by immunofluorescence in permeabilized hippocampal neurons. PrPSc was localized in the cell bodies and neurites of control and copper treated neurons (Fig. 4, A and B, respectively). A considerable increase in the fluorescence intensity was observed in neurons treated with 50 µM CuCl₂ for 24 h (Fig. 4B) compared with control neurons (Fig. 4A). This induction was observed in the cell surface staining and also in an intracellular compartment that we identified as the Golgi complex, as it colocalize with the Golgi marker giantin (36) (not shown).

Copper induces Prnp promoter activity in PC12 cells but not in C6 cells. To assess the stimulatory effect of copper on the rat Prnp promoter activity, PC12 and C6 clones stably transfected with a luciferase reporter vector driven by the rat Prnp promoter were used (10). Three different clones of each cell line were treated with 100 µM CuCl₂ for 16 h. As shown in Fig. 5A, a large increase of 20- to 40-fold in promoter activity was observed in all PC12 clones analyzed. On the other hand, copper did not induce Prnp promoter activity in any of the C6 clones studied (Fig. 5A). Cell viability under this condition was 85.49 ± 6.96% for PC12 cells and 105.32 ± 3.78% for C6 cells, as assessed by MTT assay.

PC12 clones were treated with 100 µM CuCl₂ for different incubation periods (Fig. 5B), or with different concentrations of CuCl₂ for 24 h (Fig. 5C). Because metal-inducible genes have unique dose-response curves, we used a concentration range that induces gene transcription of different mammalian genes (42, 67). A time- and concentration-dependent increase in luciferase activity was observed in the three clones studied. No promoter induction was observed in any of the C6 clones even in the presence of 200 µM CuCl₂ (data not shown). Taken together, these data demonstrate that copper activates Prnp transcription in neuronal cells suggesting that Prnp expression is regulated by copper concentration in neurons.
Fig. 3. Copper-induced PrP has a normal glycosylation pattern is proteinase K sensitive and reaches the cell surface. A: primary hippocampal neurons were exposed to 50 μM CuCl$_2$ in the presence or absence of 5 μg/ml tunicamycin for 24 h and then harvested for immunoblotting analysis of PrP. Arrowheads indicate the three distinct bands observed. Blot was probed with anti-β-tubulin antibody as loading control. B: cell lysates of hippocampal neurons untreated or treated with 25, 50, 100 μM CuCl$_2$ for 24 h were digested with 20 μg/ml PK 30 min at 37°C, and then analyzed by immunoblotting. C and D: neurons were incubated for 24 h with 50 μM CuCl$_2$ in the presence or absence of 10 nM/ml phosphatidylinositol-specific phospholipase (PIPLC). The collected medium (D) and cell lysates (C) were analyzed by immunoblotting. In all experiments PrP was detected with the anti-PrP monoclonal antibody 8H4. Molecular size markers are indicated as bars on the left side of the panel and represent 34 and 26 kDa.

Fig. 4. Cell localization of copper-induced PrP$^{C}$ in hippocampal neurons. Immunodetection of PrP$^{C}$ in cultured rat hippocampal neurons untreated (A) or treated with 50 μM CuCl$_2$ for 24 h (B) was carried out using the anti-PrP monoclonal antibody 8H4 and a secondary antibody conjugated with Alexa 555. Original magnification ×60. Scale bars indicate 10 μm.
The effect of other heavy metals, which are known to induce transcription of metal-related proteins like metallothioneins and Cu/Zn superoxide dismutase (SOD-1), was evaluated. Treatment of PC12 clones with different concentrations of CdCl₂ for 24 h increased luciferase activity in all clones studied (Fig. 6A). In contrast, no effect was observed with ZnCl₂ (Fig. 6B) treatment. In addition, we analyzed the effect of manganese, which has been demonstrated to bind to recombinant PrPC. The binding of manganese induces changes in the normal structure of PrP causing the protein to form aggregates (34, 60), and converts PrP into an abnormal form rich in \(\beta\)-sheet structure and is PK resistant (6, 34). No effect on Prnp promoter activity was observed in PC12 clones treated with 10, 50, and 100 \(\mu\)M MnCl₂ for 24 h (Fig. 6C), suggesting that manganese is unable to modulate Prnp transcription. These results indicate that there is a metal-specific regulation of Prnp expression.

Copper induction of Prnp expression is mediated by the promoter region \(-2,831\) to \(-2,150\) bp and is independent of MTF-1. Metallothionein (MT) genes are among the best studied heavy metal inducible genes, in which transcription is mediated through MREs present in multiple copies in the MT promoter region (12, 16, 27). The MRE constitutes an imperfectly conserved 12 base pair sequence motif, consisting of a highly conserved 7-base pair functional core motif \([5'-TGC(A/G)NC]\) flanked by a less conserved 5-base pair GC-rich domain (19). Sequence analysis of the upstream region of the rat Prnp revealed an inverted MRE located at position \(-2,070\) (GGGCTGTGTGCA) and two MRE-like sequences (MLS) that mismatch the consensus core motif in one nucleotide \((-2,653\ TGCGtCCCTG\text{C}; \ -2,599\ TGCGgTCACCCCT\text{G}\) (63) (Fig. 7A). To determine whether these elements are implicated in the copper-mediated induction of Prnp, a series of luciferase constructs containing progressive deletions of the Prnp promoter were transiently transfected into PC12 cells (Fig. 7A). The reporter vector containing the entire Prnp promoter (p2831), previously used to generate PC12 and C6 clones, was induced after treatment with 100 \(\mu\)M CuCl₂ for 16 h (Fig. 7B). Deletion of the region that contains the MLS1 (p2636), significantly decreased the effect of copper on promoter activity raising the possibility that MLS1 is important for copper upregulation of Prnp. No additional effect was observed when MLS2 was deleted (p2483). Deletion of the region between \(-2,483\) and \(-2,150\), which does not contain MLSs or MRE sequences, also seems to participate in copper-mediated induction of Prnp expression.

MRE sequences are recognized by the transcription factor MTF-1, which is responsible for the induction of gene transcription by heavy metals, such as zinc, cadmium, and copper (22, 45). To elucidate whether MTF-1 binds to the MLS1 in the rat Prnp promoter, an oligonucleotide corresponding to this element was used to examine the binding of PC12 nuclear proteins using EMSA. MLS1 associates with nuclear proteins
from PC12 cells in the presence or absence of 100 μM CuCl₂ for 16 h (Fig. 8A, lanes 2 and 5, respectively). These complexes disappeared when unlabeled oligonucleotide was added to the reaction mixture in 200-fold excess (Fig. 8A, lanes 3 and 6). The formation of protein-DNA complexes was not inhibited by the addition of 1,000-fold excess of a nonspecific competitor corresponding to the element for the basal transcription factor Sp1 (Fig. 8A, lanes 4 and 7). Despite the fact that MTF-1 is expressed in PC12 cells as evaluated by immunoblotting (data not shown), MLS1-nuclear protein complexes were not supershifted by an anti-MTF-1 antibody (Fig. 7B, lanes 3 and 5) that has been previously shown to supershift MTF-1 (18, 32). These data indicate that MTF-1 is not part of the formed complexes and strongly suggests that copper induction of Prnp expression is MTF-1 independent.

**DISCUSSION**

The PrPC is a normal cellular protein that has been related to prion diseases (48). Besides other physiological functions of PrPC, its involvement in copper uptake and metabolism has been largely discussed (7, 33, 47, 51). In the present work, we studied the effect of copper on the expression of PrPC in neurons. Our results indicate that copper upregulates Prnp expression in primary cultured neurons, which is observed at...
mRNA and protein levels. Prnp promoter activity in response to copper was also evaluated; a time- and concentration-dependent increase of promoter activity was observed in PC12 clones but not in C6 clones stably transfected with a luciferase reporter vector driven by the rat Prnp promoter, suggesting that Prnp expression is specifically regulated by copper concentration in neurons. The absence of stimulation of Prnp promoter activity in C6 clones is not a consequence of a nonfunctional insertion of the plasmid because it was previously demonstrated that treatment with Trichostatin A, an histone deacetylase inhibitor, increased Prnp promoter activity in all clones used in the present study (10).

Induction of PrPC expression was observed when copper was provided as CuCl2 or as a copper-glycine complex, and was prevented by cotreatment with BCS. BCS is an impermeable Cu1+-specific chelator that inhibits copper transport into the cell. This indicates that increased intracellular copper concentration might be responsible for the induction of PrPC expression by CuCl2 and copper glycine treatments. In agreement, we determined that the cellular copper content was increased by CuCl2 treatment. However, this treatment induced very low neuronal toxicity and did not induce the expression of the stress marker HSP70. The heat shock or stress response is characterized by an increase in transcription and translation of HSP70 (29). These data suggest that no stress response was induced by treatment with 50 μM Cu2+.

A recent study (59) determined that physiological concentrations of copper (5–40 μM) for 96 h decrease PrPC mRNA levels in the neuronal GN11 cells. We observed that concentrations lower than 50 μM were unable to induce the expression of PrPC, although we did not observe a decrease of PrPC in hippocampal and cortical neurons. However, we did never expose primary cultured neurons to copper treatment for >24 h. Another recent study (3) determined, with the use of cDNA microarrays, that Prnp is upregulated in fibroblast cells from mice with mutations in the ATP7A copper transporting P-type ATPase gene, which accumulate high levels of copper due to a defect in cellular copper export. This suggests that increased copper concentrations associated to dysfunction of copper metabolism may induce the expression of PrPC, supporting our findings in neuronal cells. A possible explanation for the contrasting results on Prnp modulation could be that different cell types have different responses to copper exposure, which could explain the absence of induction of Prnp promoter activity in C6 glial cells. A cell type-specific regulation has been previously observed for MTs gene expression. MT-IIA, MT-IF, and the MT-IG genes are differentially expressed in different cell lines in response to cadmium, copper, and zinc (25). The authors concluded that cell type-specific regulation of MT genes seems to be correlated to DNA methylation of cis-acting elements and the chromatin structure, and that the differential induction of MT gene expression in response to heavy metals may be related to the level of trans-acting factors present in a particular cell line. We have previously determined that Prnp expression is dependent on chromatin conformation (10). Further analysis would be necessary to test whether chromatin structure might modulate copper effects on Prnp expression.

Copper modulation of Prnp expression strongly supports a functional link between PrPC and copper. Another protein that has also been related to copper metabolism is the amyloid precursor protein (APP), which is related to Alzheimer’s disease (21). APP possesses two copper binding domains, located in its NH2 terminal region (61) and in the COOH terminal region within the Aβ domain (4), and reduces Cu2+ to Cu1+ (41, 50). As observed for PrPC, APP expression is modulated by copper concentration. Human fibroblasts overexpressing a copper efflux protein, the Menkes protein, that have severely depleted intracellular copper, show reduced APP protein levels and downregulated APP gene expression (5). In addition, APP gene is upregulated in fibroblast cells from mice with mutations in the ATP7A gene (3), which have high intracellular copper content. Overall, this evidence further supports a role for both proteins, APP and PrPC, to function in copper homeostasis (24).

In N2a cells copper induces changes in PrPC detergent solubility and intracellular retention (28). In addition, we have observed that hippocampal neurons incubated with copper
containing media produce detergent-insoluble PrP aggregates with an abnormal glycosylation pattern in which the monoglycosylated form seems to predominate, suggesting that copper alters PrP<sup>C</sup> biosynthetic folding and/or trafficking leading to accumulation of immature forms (62). Herein, we observed that in the presence of copper most of PrP<sup>C</sup> reaches the cell surface attached by a glycosyl phosphatidylinositol anchor because it was released by PIPLC treatment. This suggests that exocytic transport of PrP<sup>C</sup> to the cell surface is not prevented by copper treatment in neurons. It is known that copper induces endocytosis of PrP<sup>C</sup> (47), thus it is possible that copper may exert an effect on PrP<sup>C</sup> after it reaches the cell surface, probably during recycling or degradation. Our efforts are currently concentrated on this hypothesis.

In vitro copper converts PrP<sup>C</sup> into a detergent-insoluble and PK-resistant specie (49, 66). In addition, copper enhances PK resistance of PrP<sup>Sc</sup> (54) and facilitates restoration of PK resistance and infectivity of guanidine-denatured PrP<sup>Sc</sup> (38). This evidence strongly points to a role of copper in the conversion process of PrP<sup>C</sup> into PrP<sup>Sc</sup>. Our results showed that copper treatment that induces the expression of Prnp did not induce PK-resistant PrP specie in primary hippocampal neurons. Although we cannot eliminate the possibility that PrP could acquire protease resistance after longer exposure to elevated concentrations of copper.

Manganese is able to convert PrP<sup>C</sup> into a β-sheet rich and PK-resistant molecule (6, 20), and has a proaggregational effect inducing association of PrP oligomers to larger aggregates (34, 60). Moreover, scrapie-infected mice present a metal imbalance, characterized by systemic increase of manganese (30, 58). This evidence suggests that this metal may participate in the conversion of PrP<sup>C</sup> into PrP<sup>Sc</sup>. We evaluated whether manganese also has an effect on Prnp gene expression, and found that it was unable to regulate Prnp promoter activity in PC12 clones. This indicates that the proposed role of manganese might be exclusively a consequence of the effects of this metal on the structure of PrP and is independent of changes in Prp expression.

We also analyzed the effect of other heavy metals on Prnp promoter activity and demonstrated that cadmium strongly induced Prnp promoter in PC12 clones, whereas zinc had no effect. This is different to what is observed for metallothioneins and Cu/Zn SOD-1 genes, which are induced by copper, cadmium, and zinc (42, 67). Heavy metal-dependent induction is mediated by multiple MREs located upstream of metallothioneins genes (12, 16, 27), and by a single inverted MRE sequence in the promoter region of the SOD-1 gene (67). In the mouse, it has been also observed that the presence of a single MRE is enough to make some promoters to be target of MTF-1 (35), the transcription factor associated with metal responsiveness (22, 45). Sequence analysis of the rat Prnp promoter revealed the presence of an inverted MRE sequence and two MLSs that match the consensus sequence in six of seven positions (63). This observation raised the possibility that Prnp could be a copper-inducible gene regulated by MRE motifs similarly to metallothioneins and SOD-1 genes. Although deletion experiments suggested that MLS1 motif is important for copper upregulation of Prnp promoter activity, supershift analysis of the EMSA complexes revealed no MTF-1 binding to this motif. These results suggest that copper modulation of Prnp in neuronal cells is independent of MTF-1, which is in agreement with the absence of activation of the Prnp promoter by zinc, a well known inducer of DNA binding activity of MTF-1. As determined by immunoblot analysis, MTF-1 is expressed in PC12 cells as well as in C6 glial cells (not shown), supporting a cell type-specific regulation of Prnp expression by copper independent of MTF-1. Previously, it was shown in the human neuroblastoma cells IMR-32 that zinc does not activate the metallothionein-1 gene promoter (15). Moreover, cadmium-mediated MRE activation in this neuroblastoma cells is independent of MTF-1 (15). Taken together, these observations suggest that specific mechanisms for metal-induced transcription may occur in neuronal cells.

Our data provides new information about the regulatory mechanisms that control PrP<sup>C</sup> expression. Copper is an essential trace element that is required for the catalytic activity of a number of enzymes. The ability of copper to undergo reversible redox changes makes it a useful cofactor in redox reactions; however, the redox transition can also result in generation of reactive oxygen species by Fenton reaction, which can be toxic to cells. Thus tightly regulated copper homeostatic mechanisms are needed to control copper concentration and to prevent cellular toxicity. Metals participate directly as signals transducers in the regulation of uptake and detoxification pathways (43). The finding that copper induces Prnp expression is consistent with a role of PrP<sup>C</sup> in these pathways, and is compatible with a role for PrP<sup>C</sup> as part of a stress response mechanism. Previously, Prnp expression was shown to be upregulated by heat shock through heat-shock elements present in its promoter (−680 and −1,653 bp) (53), and sequence analysis of the promoter region of Prnp revealed the presence of two antioxidant response elements at −688 and −194 bp.

The induction of the Prnp promoter activity by copper and heat shock treatments, and the presence of putative antioxidant response elements strongly suggest that PrP<sup>C</sup> might be induced in cellular stress responses.

The finding that copper induces Prnp expression in neurons strongly supports a role of PrP<sup>C</sup> in neuronal copper homeostasis. Future experiments should consider whether this induction has an effect on the appearance and progression of prion diseases.

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