THE BLUE COPPER CERULOPLASMIN INDUCES AGGREGATION OF NEWLY DIFFERENTIATED NEURONS: A POTENTIAL MODULATOR OF NERVOUS SYSTEM ORGANIZATION

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Abstract—Ceruloplasmin (CP) is a copper-dependent ferroxidase. It regulates iron metabolism and is involved in inflammation, angiogenesis, and protection against oxidative stress. CP also modulates K⁺ channel activity in neuroblastoma cells and affects cardiodynamics of isolated hearts. Considering the presence of CP in the nervous system and the importance of iron ions and K⁺ channels in neuronal activity, we postulated a role of CP in neuronal development. This hypothesis was tested using the P19 mouse embryonal carcinoma cell line, a model of neuronal differentiation. Addition of CP to the culture medium of newly differentiated P19 neurons induced cell aggregation within 24 h. This effect was concentration-dependent half-maximal at 50 nM, and not associated with necrosis, apoptosis or changes in secretory function. Deglycosylated CP was aggregative but not denatured CP, copper salts, His₂Cu complex, or other copper enzymes or serum proteins. CP-induced aggregation was less pronounced with aging neurons and seemed not to involve K⁺ channels. Immunocytofluorescence analysis demonstrated that digoxigenin-labeled CP bound to P19 neurons and the proportion of responding neurons decreased with aging. The interaction of digoxigenin-labeled CP with neurons was half-maximal at 120 nM by enzyme-linked immunosorbent assay and displaced by unlabeled CP. Our data indicate a specific aggregative action of CP on young neurons in vitro, possibly involving CP receptors. A potential developmental role of CP in nervous system organization is thus demonstrated. © 2003 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: mouse embryonal carcinoma, neuronal migration/adhesion, nervous system development, neurotropic secretion, binding/receptor sites, patch-clamp.

Ceruloplasmin (CP) is a 132 kDa blue copper glycoprotein secreted by hepatocytes. This protein is also expressed in the nervous system (Klomp and Gitlin, 1996) as a glial glycosylphosphatidylinositol (GPI)-derivative (Patel and David, 1997; Salzer et al., 1998). CP is the major copper carrier in the plasma, binding 70–95% of circulating copper, and has ferroxidase and oxidase activities as well as antioxidant capacities (Goldstein et al., 1982; Atanasiu et al., 1998). CP was proposed to be involved in the acute phase of inflammation response (Goldstein et al., 1982; Bowman, 1993), in angiogenesis (McAulian et al., 1983), and in copper homeostasis (Bowman, 1993). CP was also reported to influence the activity of K⁺ channels in cultured neuroblastoma cells (Wang et al., 1995) and prolong the effective refractory period of isolated hearts (Atanasiu et al., 1996).

Recent studies underscored the importance of CP as a regulator of iron metabolism since this protein controls the oxidative state of iron ions and their flux in and out of cells (Mukhopadhyay et al., 1998; Harris et al., 1999; Qian and Ke, 2001). Acreruloplasminemia, a genetic deficiency of functional CP, causes iron accumulation and degeneration in brain and other organs (Harris et al., 1995; Klomp and Gitlin, 1996; David and Patel, 2000). CP oxidizes Fe²⁺ to the ferric Fe³⁺ ion species that binds to proteins (e.g., transferrin) and can be transferred across cell membranes to sustain the function of iron-dependent enzymes (Mukhopadhyay et al., 1998; Harris et al., 1999; Qian and Ke, 2001). In contrast, Fe²⁺ is not protein bound and can catalyze the production of toxic peroxidant OH radicals by Fenton reaction. The ferroxidase activity of CP in conjunction with its scavenging capacity toward reactive oxygen species (Atanasiu et al., 1998) would help decrease oxidative stress in vivo. This scenario has been repeatedly shown in our previous studies in which CP protected isolated hearts and cultured neurons against oxidative stress (Chahine et al., 1991; Atanasiu et al., 1995; Mateescu et al., 1995; Dumoulin et al., 1996; Aouffen et al., 2001). A reduced anti-oxidant effect of CP may also be linked to various neurodegenerative pathologies, such as Parkinson’s and Alzheimer’s diseases, which are associated with altered iron metabolism and oxidative stress-induced injury.

P19 mouse embryonal carcinoma cells provide an excellent cell differentiation model since they differentiate to cell derivatives of all three germ layers by apparently the same mechanisms as the normal embryonic stem cells (McBurney, 1993; MacPherson and McBurney, 1995; Skerjanc, 1999). When cultured as aggregates in the presence of 10⁻⁸ M retinoic acid (RA) over 4 days, P19 cells undergo neurodetermination. Once transferred onto tissue...
culture surfaces, these differentiated cells extend neurites within the next 24 h and continue to mature to functional neurons resembling those of the CNS (McBurney, 1993; Staines et al., 1994; Finley et al., 1996; MacPherson et al., 1997; Jeannotte et al., 1997; Parnas and Linial, 1997; Cadet and Paquin, 2000).

Considering that CP could modulate the function of neurons by influencing the activity of K⁺ channels and of iron/copper-dependent enzymes (Hartmann and Evenson, 1992; Waggoner et al., 1999; Wang et al., 1995), it was postulated that this protein could play an important role in neuronal development. In the present study, we report the aggregative action of CP on newly differentiated P19 neurons. This action was characterized with respect to CP structure, cell type, age of neuronal cultures, and the binding of CP to neurons.

EXPERIMENTAL PROCEDURES

Purification, heat-denaturation, deglycosylation, and digoxigenin (DIG) labeling of CP

The protein was purified from bovine plasma by a single chromatographic step on aminomethyl-agarose (Mateescu et al., 1999). Heat denaturation was done at 65 °C overnight. Deglycosylated CP was obtained by digestion with N-glycosidase F (New England LabSystem, Beverly, MA, USA; 3000 U/mg CP) for 24 h at 37 °C, followed by chromatography on aminomethyl-agarose (Aouffen et al., 2001). For labeling, CP was mixed at a 1:20 molar ratio with DIG (Roche Diagnostics, Laval, Quebec, Canada) in phosphate-buffered saline (PBS) adjusted to pH 8.5, and incubated for 2 h at room temperature and then 12 h at 4 °C, with continuous stirring. Unbound DIG was removed by gel filtration on Sephadex G25. CP preparations were analyzed for protein content by a microscale Bradford assay.

Cell culture

P19 cells were cultured and differentiated as described (Jeannotte et al., 1997) with slight modifications. Undifferentiated P19 cells were propagated in complete medium containing α-modified Eagle’s minimal essential medium (α-MEM; Gibco-BRL, Burlington, Ontario, Canada) supplemented with 10% heat-inactivated fetal bovine serum (Cansera International, Rexdale, Ontario, Canada), 2.5 μM penicillin and 2.5 μg/mL streptomycin. Cells were passaged every 2 days on non-coated tissue culture dishes. For neuronal differentiation, P19 cells were seeded in bacteriological-grade Petri dishes at a density of 0.9×10⁶ cells/mL, and grown into aggregates during 4 days, in complete medium containing 0.5 μM RA (Sigma-Aldrich, Oakville, Ontario, Canada). At day 4, aggregates were trypsinized with 0.025% trypsin-1 mM EDTA (Sigma-Aldrich) in PBS. The reaction was stopped by adding an equal volume of complete α-MEM medium, and the cellular suspensions were passed by pipetting and subjected to two wash-and-centrifuge cycles. Individualized cells (neurons) were transferred to gelatinized tissue culture dishes or multiwell plates (1500-1800 cells/mm²) and grown in supplemented Neurobasal medium (Neurobasal medium containing 2% v/v B27 and 0.5 mM L-glutamine; Gibco-BRL) in the absence of RA. Culture surfaces were gelatinized by incubation with 0.1% gelatin for 0.5 h at room temperature. For comparison, solutions of 2% gelatin, 10 μg/mL fibronectin, 10 μg/mL laminin or 5 mg/mL CP were also used for coating. For differentiation to fibroblasts, P19 cells were grown as monolayers for 4 days in complete medium containing RA. At day 4, cells were trypsinized, plated in non-coated tissue culture dishes and cultured in complete medium without RA. Rat hepatocytes, freshly seeded in non-coated tissue culture dishes (Haidara et al., 1999), were generously provided by Dr. F. Denizeau (Université du Québec à Montréal, Montréal, Quebec, Canada).

Treatment of cells with CP or other agents

At 3, 24 or 48 h after their transfer to tissue culture vessels, P19 neurons were incubated for another 24 h in fresh supplemented Neurobasal medium in the absence or presence of CP or other agents. Undifferentiated P19 cells, P19 fibroblasts, and rat hepatocytes were also treated with CP. In these cases, the cells were first allowed to adhere for 3 and 24 h to tissue culture surfaces in their normal serum-containing medium. Afterward, culture media were changed for supplemented Neurobasal medium containing CP. Untreated and treated cell cultures were examined for morphology, occupancy of culture surfaces, AlamarBlue dye penetration, viability, apoptosis and/or secretion of somatostatin.

The His₃Cu reagent was obtained by adding L-histidine up to 6.4 mM in a 0.9% w/v NaCl solution containing 7.9 mM CuCl₂ and adjusted to pH 7.4 (Sarkar et al., 1993). Laccase and albumin were from Sigma-Aldrich and Roche Diagnostics, respectively. Bovine serum amine oxidase was purified as per Mateescu et al. (1999).

Cell morphology

Visual morphologic evaluation was done with a Nikon TMS microscope, using phase-contrast objectives and a Nikon F70 camera. Photographs were taken at 100× magnification with Kodak Technical Pan films.

Occupancy of culture surfaces

A transparent grid made of 25 mm² squares was placed over cell photographs developed as 10.5×15 cm pictures in order to count the “empty” squares defined as having less than 50% of their surface occupied by cells. Three different pictures were taken per dish to provide a mean number of empty squares for the dish. Numbers of empty squares in treated cultures were expressed relatively to untreated cultures. Because of its simplicity and rapidity, this grid method was adopted after correlation of the results by image analysis with Un-Scan-it software (SilkScientific Inc.).

AlamarBlue dye penetration

Day 4 P19 neurons were plated in 24- or 48-well plates. After treatment with CP, cells were incubated for 5 h with 500 μM of a fresh solution containing nine parts of supplemented Neurobasal medium and one part of AlamarBlue dye (Medicorp Inc., Montreal, Quebec, Canada). The incubation media were then monitored for dye reduction by fluorescence (exc. 540 nm, em. 590 nm). The AlamarBlue dye enters into cells where it is metabolically reduced and, with time, the reduced form accumulates in the culture medium.

Cell viability

Cells were trypsinized in PBS containing 0.25% trypsin and 1 mM EDTA (Sigma-Aldrich) for 10 min at 37 °C. Digestion was stopped by the addition of one volume of complete α-MEM medium. Cells were passaged by pipetting, stained by the addition of two volumes PBS containing 20 μg/mL propidium iodide and 3 μg/mL acridine orange, and counted with a hemacytometer under a microscope set up for fluorescein fluorescence. Live and dead cells were respectively stained green and red.

Apoptosis analysis

Day 4 P19 neurons were plated in 24-well plates, treated with CP and then analyzed for DNA fragmentation with a Nucleosome
enzyme-linked immunosorbent assay (ELISA) kit (Oncogene Research Products; Calbiochem, Boston, MA, USA). Briefly, cells were washed with PBS, lysed in 350 μL lysis buffer (included in the kit and freshly complemented with phenylmethylsulfonyl fluoride to a final concentration of 0.2 mM), incubated for 30 min on ice and centrifuged. Supernatants were then assayed for nucleosome content as per manufacturer’s instructions. Cells were also examined for chromatin condensation after staining with Hoechst 33258 (Sigma-Aldrich). For this assay, cell monolayers were incubated for 15 min at room temperature with the dye added at a final concentration of 50 μg/mL in the culture medium, washed twice with PBS, and observed by fluorescence microscopy. Alternatively, cells were individualized with 0.25% trypsin-1 mM EDTA in PBS before staining to permit the counting of fluorescing cells.

**Secretion of somatostatin**

Day 5 P19 neurons, with or without pre-treatment for 24 h with CP, were incubated for 1 h in a fresh provision of supplemented Neurobasal medium containing the protease inhibitors aprotinin (Roche Diagnostics; 30 μg/mL) and soybean trypsin inhibitor (Sigma-Aldrich; 100 μg/mL). They were then challenged with 50 mM KCl or an equivalent volume of water (control) for 1 h. Culture media were then analyzed for somatostatin by radioimmunoassay (Cadet and Paquin, 2000), and secretion values normalized to the number of cells in dishes. Results were compared by unpaired Student’s t-test.

**Binding of DIG-CP to cells**

For immunocytofluorescence studies, cells were seeded onto poly-α-lysine-coated cover glasses or in gelatinized wells of tissue culture plates, and grown in supplemented Neurobasal medium (P19 neurons) or in complete α-MEM medium (P19 undifferentiated cells and fibroblasts). Cells were then washed twice in a pH 7.4 Hanks’ balanced salt solution containing 0.1% Tween-20 (HBSS-T), fixed with 4% paraformaldehyde in PBS for 30 min, blocked with 1% bovine serum albumin in HBSS-T for 1 h, and incubated with 1000 nM DIG-CP in HBSS-T for 2 h at room temperature. Unbound DIG-CP was washed off and cells incubated with a fluorescein-conjugated anti-DIG antibody (10 μg/mL in HBSS-T; Roche Diagnostics) for 1 h at room temperature. Cells were examined by fluorescence with a Zeiss inverted microscope (Carl Zeiss, Germany) interfaced with a digital camera (Sony DYC-950P) and a Northern Eclipse software (EMPIX Imaging Inc., Mississauga, Ontario, Canada). Evaluation of CP binding to cells was done by an indirect ELISA. Day 4 P19 neurons and undifferentiated cells were grown in gelatinized 96-well tissue culture plates in their respective culture media. Cells were then slightly fixed in PBS containing 0.1% glutaraldehyde (this fixation helped cells in withstanding solution changes), blocked with 1% bovine serum albumin in HBSS-T, and then incubated for 2 h, at room temperature, with 100 μL HBSS-T containing various concentrations of DIG-CP (0–1000 nM), in the absence or presence of unlabeled CP. Unbound protein molecules were removed and cells reincubated for 1 h in 100 μL HBSS-T containing a peroxidase-conjugated anti-DIG antibody (2 μg/mL; Roche Diagnostics). The bound antibody was assayed spectrophotometrically (SPECTRAmax Gemini; Molecular Devices, Sunnyvale, CA, USA) at 412 nm by incubating cells for 30 min with 150 μL of a BM-blue peroxidase substrate solution (Roche Diagnostics). Control studies demonstrated that the amounts of peroxidase-conjugated antibody and peroxidase substrate were not limiting. The ELISA results were normalized for cell protein content determined by a microscale Bradford assay.

**Electrophysiologic studies**

Voltage-dependent K⁺ channel currents of P19 neurons were recorded using the whole-cell configuration of the patch-clamp technique described for neuroblastoma cells (Wang et al., 1995).

**RESULTS**

**CP has a saturable pro-aggregative effect on neurons**

Mature neurons respond to secretagogue stimulation, such as K⁺-induced membrane depolarization, by massively releasing neurotransmitters and neuropeptides in the extracellular milieu. Newly differentiated P19 neurons develop their peptidergic properties in a stepwise manner. They acquire first the capacity to synthesize neuropeptides and, as they mature, the capacity to release these substances under K⁺ stimulation (Cadet and Paquin, 2000). In view of CP effect on K⁺ channel activity in neuroblastoma cells (Wang et al., 1995), we reasoned that this protein could perhaps help in accelerating the maturation of P19 neurons. We therefore exposed day 4 P19 neurons to 0.5 mg/mL (3.8 μM) CP, a concentration value between those found in the plasma under physiological (0.1–0.3 mg/mL) and inflammatory (0.7–0.9 mg/mL) conditions. Unexpectedly, the protein had a strong aggregative effect on cells. While neurons cultured in the absence of CP formed well-spread cell monolayers, those treated for 24 h with the protein formed compact cell aggregates that adhered loosely to the culture surface but still exhibited interconnecting neurites (Fig. 1A).

The pro-aggregative effect of CP was concentration-dependent up to 0.15 μM and leveled at higher concentrations (Fig. 1B). Aggregation observed by morphologic changes was stable even at concentrations as high as 3.8 μM (not shown). Aggregation was also quantified by estimating the increase in cell-free surface accompanying neuron packing. The concentration-response curve obtained with this approach (Fig. 1C) matched the morphologic results (Fig. 1B), with about 0.05 μM CP achieving 50% of the aggregative effect. We also used the Alamar-Blue dye to assay aggregation. This dye must penetrate cells to get metabolically reduced and yield fluorescence. As aggregation impairs the access of extracellular substances to cells, there was less dye reduction in aggregated as compared with non-aggregated cultures. The monitoring of dye reduction confirmed the concentration-dependent and saturable pro-aggregative effect of CP (Fig. 1D).

**CP does not compromise neuronal viability and function**

CP concentrations ≥4 μM were toxic to isolated rat hearts perfused in serum-free conditions (Chahine et al., 1991; Atanasiu et al., 1995) and those over 1 μM, lethal to cultured neuroblastoma cells (Wang et al., 1995). We therefore evaluated the viability of CP treated-neurons to determine whether aggregation was associated with cytotoxicity. When aggregated neurons were individualized by trypsinization and stained with viability dyes, almost all of them incorporated acridine orange but not propidium io-
dide, indicating intact plasma membranes and viability (not shown). This held for CP concentrations up to 7.6 µM. We also tested whether the protein could trigger apoptosis, a programmed cell death used by the developing nervous system to control the number of neurons (Honig and Rosenberg, 2000). Both Hoechst dye and nucleosome assays demonstrated that CP, up to 3.8 µM, had no pro-apoptotic effect on P19 neurons. Indeed, less than 5% cells in untreated and CP-treated cultures were stained by Hoechst dye, indicating no induction of chromatin condensation by the protein. In addition, all cultures showed similar low nucleosome levels (approximately 4.2 nucleosome units per 10^6 cells), indicating no induction of DNA fragmentation either. In contrast, nucleosome levels were about five-fold higher in apoptotic cell controls, including P19 neurons irradiated with ultraviolet light for 15 min and hepatocytes treated with 0.16 ng/mL β-transforming growth factor for 24 h.

Newly differentiated neurons (days 5–8) synthesize and constitutively secrete basal levels of neuropeptides, such as somatostatin (Cadet and Paquin, 2000). CP-treated neurons remained capable to secrete somatostatin (Table 1). Their basal secretory rate was about half that of untreated or buffer-treated neurons (Table 1, without KCl). This decrease was likely due to a slower diffusion of somatostatin out of the cell aggregates since the diffusion of AlamarBlue into these assemblages was also impaired by a similar factor (Fig. 1D). As reported before (Cadet and Paquin, 2000), K^+ did not enhance somatostatin secretion in newly differentiated neurons (Table 1, none agent).

**Fig. 1.** Aggregative effect of CP on P19 neurons. Day 4 P19 neurons were plated in gelatin-coated tissue culture dishes and incubated for 24 h with various concentrations of CP (added 3 h after plating). (A) Phase-contrast micrographs of P19 neurons not treated or treated with 3.8 µM CP. Incubation of cells with CP’s buffer (control) had no morphological effect. (B-D) Concentration dependency of CP neuroaggregative effect assessed by three methods. (B) Culture morphology: 0, + or ++ means no, slight or moderate restriction in cell spreading, respectively; ++++, high restriction in cell spreading with a tendency to form aggregates; ++++, intense cell aggregation. (C) Measurement of cell-free surface in neuronal cultures. The values shown for one study are reported as means±S.D. of three different pictures taken for each CP concentration. Three independent studies gave the same results. (D) Cellular reduction of AlamarBlue dye. At the end of CP treatments, the dye was added to culture media at 10% v/v final concentration and, 5 h later, assayed fluorimetrically for reduction. The values are expressed as percentages of untreated cells, and reported as means±S.D. for triplicate determinations. The results shown for one experiment are representative of three independent studies.
Table 1. Secretion rate of somatostatin in non-aggregated and CP-aggregated P19 neurons

<table>
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<tr>
<th>Agent</th>
<th>pg/2 h/30,000 neurons</th>
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<tr>
<td></td>
<td>Without KCl</td>
</tr>
<tr>
<td>None</td>
<td>11.2±3.9</td>
</tr>
<tr>
<td>CP’s buffer</td>
<td>9.6±1.1</td>
</tr>
<tr>
<td>CP 0.2 mg/mL</td>
<td>5.3±1.8</td>
</tr>
<tr>
<td>CP 0.5 mg/mL</td>
<td>3.4±1.7</td>
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</table>

Day 5 P19 neurons were incubated for 48 h in supplemented Neurobasal medium in the presence of the indicated agent. CP, but not its buffer, induced neuroaggregation. Culture media were replenished and neurons incubated for 1 h for equilibration and then for another 1 h in the absence or presence of 50 mM KCl as a secretagogue. Culture media were collected for determination of somatostatin by radioimmunoassay and data normalized for the number of cells. Results are expressed as the means±S.D. for four independent studies, each one involving three dishes per agent. CP’s buffer was 0.1 M potassium phosphate, pH 7.4. NS indicates no significant difference with corresponding cells not exposed to KCl, using P<0.05 as the significance level.

Likewise, CP-treated cells were not stimulated by K+ (Table 1), indicating a similar secretory maturation state for untreated and treated neurons.

The effect of CP on the morphology of aging neurons and non-neuronal cells

Since newly differentiated P19 neurons continue to mature in vitro, we determined whether they exhibit an age-dependent response to the pro-aggregative influence of CP. P19 neurons begin to adhere to the substratum 3 h after plating and to develop a neuritic network in the following 24 h. When treated with CP at 24 or 48 h after plating, instead of 3 h, neurons still aggregated despite the presence of neurites. However, aging decreased neuron sensitivity toward CP action since half maximal aggregation occurred at 0.33 and 0.62 μM, respectively, as compared with 0.05 μM (Fig. 2). Interestingly, many aggregates remained interconnected by long and thick processes (as in Fig. 1A), suggesting that CP did not directly cause neurite retraction. Addition of CP at more than 72 h after plating did not induce aggregation or neurite retraction (not shown). Treatment with 3.8 μM CP, a saturating neuroaggregative concentration, did not induce the aggregation of undifferentiated P19 cells, P19-derived fibroblasts, and rat hepatocytes (not shown), indicating a cell type specific phenomenon.

Culture substrate does not influence CP-induced neuroaggregation

Cell migration and adhesion are controlled by interactions with the extracellular matrix. To test whether CP-induced neuroaggregation involves changes in cell–matrix interactions, we examined the effects of CP with different culture substrata. When plated onto fibronectin, laminin, poly-lysine or surfaces coated with high concentrations of gelatin, P19 neurons still extensively aggregated (++) in the presence of 3.8 μM CP (not shown). Also, when CP itself was tested as a coating material, it had an aggregative effect similar to that of soluble CP (not shown). It thus appeared that CP-induced neuroaggregation was matrix type independent with respect to exogenously provided matrices.

Denatured CP, copper ions, other copper enzymes and serum proteins do not induce neuroaggregation

CP loses its blue color and as much as 50% of its copper content upon heat denaturation (Dumoulin et al., 1996). Several but not all actions of CP strictly depend on its native form. For example, native CP, but not the heat-denatured protein, has ferroxdase and oxidase activities, protects isolated hearts in oxidative stress (Chahine et al., 1991; Mateescu et al., 1995; Dumoulin et al., 1996), or modulates K+ channel activity in neuroblastoma cells (Wang et al., 1995). In contrast, heat-denatured CP is as effective as native CP as a class III antifibrillatory agent on heart (Atanasiu et al., 1996) or as a free radical scavenger in vitro (Atanasiu et al., 1998). On the other hand, deglycosylation does not compromise the enzymatic activities of CP nor its ex vivo cardio- and neuroprotective actions in oxidative stress (Aouffen et al., 2001). In the present study, heat-denatured CP, even at high concentrations, failed to induce aggregation (Fig. 3). In contrast, deglycosylated CP was still effective although less potent, with half-maximal aggregation occurring at about 10-fold higher concentration as compared with the native protein (Fig. 3). The results indicate that the protein must maintain its native conformation to some degree to be neuroaggregative, and the presence of carbohydrates, although not essential, could have a potentiating effect such as increasing the protein stability and/or its binding affinity to potential receptors.

CP contains six, and maybe an additional number of less firmly bound, copper atoms per polypeptide chain (Calabrese et al., 1981; Zaitseva et al., 1996). Neuroaggregation could be related to the release of CP’s copper ions into the cellular environment or their direct transfer to...
cells by means of CP–cell interactions. However, cell treatment with copper salts at copper concentrations equivalent to those found in CP did not induce neuroaggregation (Table 2). Histidine is a physiological copper transporter found in serum (Sarkar et al., 1993). Copper complexed as His$_2$Cu is blue, like the three type-I copper atoms of CP. It can be taken up by cells and incorporated into intracellular cuproproteins (Dameron and Harris, 1987; Katz and Barnea, 1990; Hilton et al., 1995). His$_2$Cu used at copper concentrations identical to or even higher than found in CP did not induce neuroaggregation (Table 2). Copper salts were toxic to P19 neurons at high micromolar concentration while His$_2$Cu was not (Table 2), underlining the bio-compatibility of the latter. Therefore, neither free nor histidine-coordinated copper ions were pro-aggregative by themselves. However, we cannot rule out that they could still have a role in aggregation via CP-mediated transfer across neuronal membranes.

CP action on P19 neurons was also compared with that of other copper enzymes and serum proteins (Table 2). Laccase (64 kDa), which has a blue copper center

**Table 2.** Effects of copper ions, copper enzymes and serum proteins on the morphology of P19 neurons

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration (µM)</th>
<th>Morphology</th>
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<tbody>
<tr>
<td>CuSO$_4$, CuCl$_2$</td>
<td>1.14–49.2</td>
<td>No aggregation</td>
</tr>
<tr>
<td></td>
<td>246</td>
<td>Cell death</td>
</tr>
<tr>
<td>(His)$_2$Cu complex</td>
<td>23–390</td>
<td>No aggregation</td>
</tr>
<tr>
<td>Laccase</td>
<td>0.79–7.9</td>
<td>No aggregation</td>
</tr>
<tr>
<td>Bovine serum amine oxidase</td>
<td>0.28–0.56</td>
<td>No aggregation</td>
</tr>
<tr>
<td></td>
<td>1.1</td>
<td>Cell death</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>0.3–7.5</td>
<td>No aggregation</td>
</tr>
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</table>

Day 4 P19 neurons were plated on gelatin-coated dishes and incubated for 24 h with different concentrations of the indicated agent. The morphology of cell cultures was examined under the microscope. Cell death was characterized by cellular shrinkage or lysis. Assuming six copper atoms in CP structure, a concentration of 22.8 µM copper ions simulated a complete release of copper atoms from 3.8 µM CP.

(Messerschmidt and Huber, 1990), was not pro-aggregative. Serum amine oxidase (180 kDa), a circulating non-blue copper protein which can modulate K$^+$ channel activity (Wu et al., 1996), did not induce aggregation either. It even caused cell death when used at 1.1 µM while CP up to 7.6 µM did not. Cell death can be related to the production of cytotoxic H$_2$O$_2$ and aldehydes by amine oxidase activity (Averill-Bates et al., 1993). Serum albumin which can transport copper ions among other substances and act as an antioxidant, although less potently than CP (Dumoulin et al., 1996; Atanasiu et al., 1998), also failed to induce aggregation (Table 2). In addition, albumin at 3.6 µM did not inhibit CP-induced neuroaggregation (not shown). The aggregative action was thus specific to CP and not due to a general protein effect.

**CP binds to P19 neurons**

The specific CP neuroaggregative effect prompted us to examine the presence of neuronal receptors for this protein. Because there was no antibody available for detection of the bovine protein, we used DIG-labeled CP with an anti-DIG antibody to monitor the interaction of CP with P19 neurons. MALDI-TOF mass spectrometry analysis of DIG-CP, as described by Boivin et al. (2001), revealed the incorporation of two DIG molecules per CP molecule (S. Boivin, unpublished data). This slight modification did not change the neuroaggregative properties of CP. Fig. 4A shows that DIG-CP bound to neurons but not to undifferentiated cells. The binding was saturable (Fig. 4A) and displaced by unlabeled CP (Fig. 4B), indicating a specific interaction and not merely adsorption of the DIG moiety. The value of 0.12 µM for 50% binding (Fig. 4A) resembled values for half maximal aggregation (0.05 µM for day 4 neurons, Fig. 1, and 0.33 µM for day 5 neurons, Fig. 2). Immunocytofluorescence studies with CP-DIG confirmed the capacity of CP to interact with neurons (Fig. 4C). In line with the neuroaggregation results (Fig. 2), the proportion of immunoreactive cells decreased with aging as indicated by comparing the response of day 4, day 5 and day 6 cells (Figs. 4C–E). Noteworthy, the immunoreactive signal seen with neurons was displaced by an excess of unlabeled CP (not shown), and a very faint immunoreactive signal, if any, was detected with P19 fibroblasts and undifferentiated cells (not shown).

**Electrophysiological studies**

In view that CP depolarized neuroblastoma cell membranes by acting on K$^+$ channels (Wang et al., 1995), we tested whether CP-induced neuroaggregation could be mediated by a change in the polarization status of neuronal membranes using the whole-cell configuration of the patch-clamp technique. Depolarization of P19 neurons at days 5, 6, 7 and 8 of differentiation increased the activity of voltage-dependent K$^+$ channels (not shown). Addition of CP to the perfusing extracellular buffer had no effect on these currents, indicating that K$^+$ channels were not involved in triggering CP aggregative action. Different electrophysiological responses of P19 neurons and neuroblastoma cells (Wang et al., 1995) to CP challenge could be
due to differences in K⁺ channel expression profile and/or cell maturation state.

**DISCUSSION**

This study demonstrates, for the first time, that CP has a pro-aggregative action on newly differentiated neurons *in vitro*, adding to the apparent multifunctional character of the protein. This action is specific for CP in a concentration-dependent and saturable fashion (Fig. 1). All other copper enzymes and plasma proteins, like laccase, serum amine oxidase and albumin which share some properties with CP, failed to induce neuronal aggregation (Table 2), eliminating the possibility of a general protein effect. While the aggregation was less potent with deglycosylated CP, it was abolished by heat-denaturation of the native CP (Fig. 3), emphasizing the conformational exigencies regarding the catalytic sites, copper-binding sites and/or folding pattern for the CP effect. Finally, the aggregative effect appears to be cell-type specific since it was only observed with newly differentiated neurons, but not with undifferentiated cells, fibroblasts or hepatocytes. Although CP was reported to reduce adhesion of leukocytes and BHK cells on plastic as well as leukocyte adhesion on endothelial cells (Curtis and Forrester, 1984; Broadley and Hoover, 1989), the reduced adhesion was seen at higher concentrations of CP than those promoting neuroaggregation. The specific and potent neuroaggregative action of CP in conjunction with its synthesis in the nervous system (David and Patel, 2000) raises the possibility that CP may participate in nervous tissue organization in general and neuronal development in particular.

The detailed mechanism underlying the aggregative action of CP on newly differentiated P19 neurons remains to be elucidated. The involvement of CP receptors de-
serves special attention since the protein binds to P19 neurons with a half-binding concentration of 0.12 μM compatible with its pro-aggregative concentrations. Our results are in agreement with the study of Orena et al. (1986) reporting the existence of CP binding sites in brain membrane preparations. The immunocytochemical analysis of Mollgard et al. (1988) also raised a possible association of CP with neurons in the developing human brain. An intriguing finding in our study was that cell aging decreased the capacity of P19 neurons to bind CP, suggesting an age-dependent decrease of CP receptors in differentiated neurons. The existence of CP binding sites was also reported in other cell and tissue preparations, including blood cells, aorta, heart and liver, with K₅₀ values ranging from 0.01–10 μM (Barnes and Frieden, 1984; Stevens et al., 1984; Kataoka and Tavassoli, 1985; Puchkova et al., 1997). Potential neuronal receptors could differ from hepatic receptors since CP had no aggregative effect on hepatocytes.

Although the present study was initiated with the assumption that CP could accelerate the establishment of evocable secretion in P19 neurons by acting on K⁺ channels, patch-clamp analysis indicated that CP-induced neuroaggregation seemed to be not triggered by a change in K⁺ channel activity.

The physiological significance of the neuroaggregative effect of CP cannot be fully understood at the moment but some speculations are offered. The age-dependent effect of CP may have relevance to the stepwise establishment of the laminar architecture and neuronal connectivity of the developing brain. Different brain areas originate from an array of cell layers assembled during development (Hatten, 1999). After differentiation, immature neurons migrate radially from germinative zones to neural laminae, and tangentially across laminae to form compact neuronal layers. Once at destination, they grow axons to establish synaptic contacts with targets. Neurons migrate along glial fibers (the counterparts of astrocytes in the embryonic brain) and on neuritic cables assembled by the first batches of migratory neurons (Hatten, 1999). It is thus tempting to propose that neuronal migration along glial fibers could implicate interaction between CP receptors on neurons and CP molecules anchored on glial fibers, perhaps by a GPI group like GPI-CP expressed by mature glial cells (Patel and David, 1997; Salzer et al., 1998).

The fact that CP can induce aggregation of neurite-bearing neurons and at the same time leave long neuritic connectivities between aggregates (Fig. 1A) is also compatible with the idea of CP assisting neuron migration on neuritic cables. Potential sources of CP are glial cells but also the cerebrospinal fluid which contains about 0.01 μM of the protein (Hartard et al., 1993). After migration, CP could also modulate the compactness of brain layers since neurons expressing CP receptors could interact with astrocytes, Schwann cells and brain fibroblasts expressing GPI-CP (Patel and David, 1997; Salzer et al., 1998).

In humans and mice presenting aceruloplasminemia, there was no identified case of neuromigration disorder although this specific aspect could have been overlooked.

The main defect observed was iron accumulation secondary to the lack of ferroxidase activity (David and Patel, 2000; Qian and Ke, 2001; Patel et al., 2002). However, this does not discredit our hypothesis of CP involvement in neuromigration since the presence of many other endogenous substances, including proteases and their inhibitors, growth factors, adhesion proteins, and chemotactic substances (Hatten, 1999; Yoshida and Shiosaka, 1999), may compensate for the absence of CP. Interestingly, CP knockout mice showed deficits in motor coordination that were associated with a loss of brainstem dopaminergic neurons (Patel et al., 2002). The deficit in this neuron type could be due to cell mortality induced by iron mediated-oxidative stress, as suggested by the authors, but it could also result from the disorganized neuronal aggregation and migration due to CP deficiency at specific times during development.

In conclusion, CP was shown to have a pro-aggregative action on newly differentiated neurons in vitro. Although the mechanism responsible for aggregation is not known yet, it may involve specific receptors since CP binds to these neurons. The decreasing sensitivity of neurons toward CP aggregative effect with aging suggests that the protein could have a role in early organization of the developing nervous system. To reactivate this role of CP in adult degenerating brains would be a novel approach to reconstruct nervous tissues disaggregated by disease.

The neuroaggregative action of CP adds to other properties of the protein as a modulator of iron/copper metabolism, an antioxidant, and a modulator of neuronal and cardiac functions.

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