Glycosylphosphatidylinositol-anchored Ceruloplasmin Is Required for Iron Efflux from Cells in the Central Nervous System*

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Ceruloplasmin (Cp) is a ferroxidase that converts highly toxic ferrous iron to its non-toxic ferric form. A glycosylphosphatidylinositol (GPI)-anchored form of this enzyme is expressed by astrocytes in the mammalian central nervous system, whereas the secreted form is expressed by the liver and found in serum. Lack of this enzyme results in iron accumulation in the brain and neurodegeneration. Herein, we show using astrocytes purified from the central nervous system of Cp-null mice that GPI-Cp is essential for iron efflux and not involved in regulating iron influx. We also show that GPI-Cp colocalizes on the astrocyte cell surface with the divalent metal transporter IREG1 and is physically associated with IREG1. In addition, IREG1 alone is unable to efflux iron from astrocytes in the absence of GPI-Cp or secreted Cp. We also provide evidence that the divalent metal influx transporter DMT1 is expressed by astrocytes and is likely to mediate iron influx into these glial cells. The coordinated actions of GPI-Cp and IREG1 may be required for iron efflux from neural cells, and disruption of this balance could lead to iron accumulation in the central nervous system and neurodegeneration.

Mechanisms to maintain iron homeostasis at the cellular level are crucial for the viability of cells. Excess or inappropriately shielded cellular iron can lead to cell death. The effects of this toxicity are especially noticeable in the brain, spinal cord, and other parts of the central nervous system (CNS), because the mature CNS lacks regenerative capabilities. Although iron is essential for a variety of biological functions, such as oxygen transport, mitochondrial respiration, and DNA synthesis, it can generate highly toxic free radicals because it is a transition metal; thus, shielded cellular iron can lead to cell death. The effects of this enzyme result in iron accumulation in the brain and neurodegeneration. Herein, we show using astrocytes purified from the central nervous system of Cp-null mice that GPI-Cp is essential for iron efflux and not involved in regulating iron influx. We also show that GPI-Cp colocalizes on the astrocyte cell surface with the divalent metal transporter IREG1 and is physically associated with IREG1. In addition, IREG1 alone is unable to efflux iron from astrocytes in the absence of GPI-Cp or secreted Cp. We also provide evidence that the divalent metal influx transporter DMT1 is expressed by astrocytes and is likely to mediate iron influx into these glial cells. The coordinated actions of GPI-Cp and IREG1 may be required for iron efflux from neural cells, and disruption of this balance could lead to iron accumulation in the central nervous system and neurodegeneration.

Experimental Procedures

Iron Influx/Efflux Studies—Astrocytes were purified from the brains of neonatal wild-type (Cp+/+) and Cp-null mice (Cp−/−) obtained from littermates and cultured as described previously (17). The Cp-null mice were generated in this laboratory as reported previously (6). Cultured astrocytes were plated on poly-l-lysine (Sigma)-coated 24-well plates 2 days before the experiment at a density of 3 × 10^5 cells/well. Cells were washed with serum-free Dulbecco's modified Eagle's medium (DMEM; Invitrogen) twice and incubated in DMEM containing vitamins and penicillin/streptomycin for 1 h at 37 °C to remove any transferrin-bound iron.

Influx Study—After 1 h, DMEM was replaced with Sato's modified chemically defined serum-free medium (18) without transferrin. Radio-

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The abbreviations used are: CNS, central nervous system; Cp, ceruloplasmin; CSF, cerebrospinal fluid; GPI, glycosylphosphatidylinositol; Dcytb, duodenal cytochrome b; DMT1, divalent metal transporter IREG1; RT-PCR, reverse transcription PCR.
labeled iron (transferrin) was added to cells, and non-labeled FeCl₃ (total of 40 μg/ml) and t-ascorbate (Sigma) was added to keep iron in its ferrous form (molar ratio of FeCl₃ to t-ascorbate was 1:4). After different culture periods, cells were treated with PRONASE protease (Calbiochem) for 1 h at 4°C to remove membrane-bound iron and then lysed in 1% NaOH. Additional experiments with EDTA (500 μg/ml) and PRONASE treatment gave results similar to those of PRONASE alone (data not shown). The amount of radioactivity bound nonspecifically to the cells was estimated by adding the media containing radiolabeled iron to culture wells and removing it within 1–2 min, washing the wells, and measuring with a γ-counter (Amersham Biosciences). This radioactivity level, which was found to be extremely low, was considered background value and was subtracted from all values at each data point. This value is considered zero at the 0-h time point. Cells were cultured in 5% CO₂ at 37°C in the media containing radiolabeled iron until the desired time points (i.e., 12, 24, and 48 h). Sister cultures were treated in the same manner without radioactive iron, and viable cell numbers were estimated by trypan blue exclusion. The amount of radioactivity that was taken up by the cells was converted into picomoles of iron using a standard graph and normalized to value per 10⁶ cells. The standard graph was plotted using counts per minute versus serial dilution of 1 μl of ⁵⁹FeCl₃.

**Efflux Study**—Cultured astrocytes in 24-well plates were washed and incubated in serum-free medium for 1 h, as was done for the influx study. Cells were then loaded with medium containing radiolabeled iron for 24 h (same condition as above). After 24 h, cells were washed twice with DMEM, and serum-free Sato’s chemically defined medium without transferrin was added to the cultures. At each time point (0, 12, 24, and 48 h) cells were detached, pelleted, and lysed in 1% NaOH. In addition, a 200-μl aliquot of culture medium from each time point was collected to measure the amount of iron released into the medium. Radioactivity in both cell pellet and culture medium was measured. All radioactivity measurements at each time point were done in quadruplicate and repeated in three separate experiments. Results are shown as mean ± S.E. Two-sample Student’s t test was used to determine statistical significance.

**RT-PCR**—Total RNA was purified from rat neonatal astrocyte cultures using a RiboPure kit (Ambion) following the manufacturer’s protocol. RT-PCR was performed using the GeneAmp RNA PCR kit (PerkinElmer Life Sciences). Primers were used as follows: DMT1 for, 5′-ACCGCCAATAGGGATGACTT-3′; DMT1_rev, 5′-GCGCAAGGCAAGAAGTTGCTT-3′; IREG1 for, 5′-TCGCTCTGGTGTCTGTGCTGTGCTTTG-3′; IREG1_rev, 5′-CTAAGGGTGGGCTGCCCAGGATGAC-3′; Dcytb_rev, 5′-CGAGGCGGCTGGGCACAGAA-3′ (Invitrogen custom-made primer). PCR was performed under the following conditions: step 1, 2.5 min at 95°C for one cycle; step 2, 45 s at 95°C, 45 s at 61.5°C (58°C for IREG1 and 60.2°C for Dcytb), and 1.5 min at 72°C for 35 cycles; and step 3, 7 min at 72°C for one cycle. RT-PCR for glyceraldehyde-3-phosphate dehydrogenase was performed as the equal use of RNA according to the conditions described previously (19).

**Western Blotting**—Cultured neonatal rat astrocytes were washed and pelleted. Total proteins were extracted with 1% Nonidet P-40 (Sigma), 1% sodium deoxycholate (BDH Chemicals), 2% SDS, 0.15 M sodium phosphate, pH 7.2, 2 mM EDTA, containing a mixture of protease inhibitors (Roche Diagnostics). Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane (Bio-Rad), and incubated with anti-IREG1 (1:4000; Alpha Diagnostics) or anti-DMT1 (1:4000; NRAMP24-S, which recognizes DMT1 with and without IRE, Alpha Diagnostics), or antiactin polyclonal antibodies for loading control (Santa Cruz Biotechnology). Blots were washed and incubated with horseradish-conjugated IgG (1:2000; Jackson Immunoresearch). Antibodies were detected with an enhanced chemiluminescence kit (PerkinElmer Life Sciences).

**Immunocytochemistry**—Purified neonatal rat astrocytes were plated on PLL-coated round glass coverslips and cultured in DMEM containing 10% fetal bovine serum. Cells were washed in Hank’s balanced salt solution with anti-ceruloplasmin monoclonal antibody (1A1; 1:100) for 30 min at room temperature. Cells were washed, and rhodamine-conjugated goat anti-mouse IgG was added for 30 min. Cells were fixed in acetic acid/ethanol (5:95 v/v) at –20°C for 20 min. Permeabilized cells were stained with either anti-IREG1 or anti-DMT1 polyclonal rabbit antibodies (1:200; Alpha Diagnostics) for 30 min at room temperature visualized by fluorescein-conjugated goat biotin by biotinylated rabbit IgG (all secondary antibodies from Jackson ImmunoResearch). Confocal microscopy was used to generate 0.8-μm sectioned images of cells.

**Coimmunoprecipitation Assay**—Purified rat astrocytes were soni-
GPI-Cp Is Required for Iron Efflux from the CNS

Although there has been an increase in our understanding of iron transport and regulation of iron levels in hematopoietic tissue, macrophages, and enterocytes in the gut, there is little direct evidence for the molecular mechanisms underlying iron transport across neural cell membranes in the CNS (reviewed in Ref. 22). Once iron gets past the endothelial cells in the CNS, its uptake into neural cells could occur via transferrin and/or non-transferrin-mediated mechanisms. Transferrin receptors are expressed by oligodendrocytes and neurons in the CNS, whereas their expression in astrocytes has been more difficult to detect, although some recent studies suggest its presence in astrocytes (23). However, transferrin levels in the CSF, which reflects the amount available to CNS tissue, is extremely low (about 1% compared with serum; 0.1–0.28 \( \mu \)mol/L in humans (24)), suggesting that under normal conditions, transferrin-mediated uptake may not be significant, particularly in astrocytes. This is further supported by the findings in hypotransferrinemic mice, which show a normal distribution of iron in the brain (25, 26). These data suggest that non-transferrin-mediated mechanisms are likely to be involved in iron influx into cells in the brain. We show here that astrocytes can indeed take up iron though non-transferrin-mediated mechanisms. We also show that astrocytes express both the divalent metal transporter DMT1, which has specificity for divalent metals, including ferrous iron, and the ferric reductase Dcytb, indicating a role for these molecules in mediating iron transport across neural cell membranes in the CNS (reviewed in Ref. 28) possibly indicating a unique homeostatic mechanism at either the level of the endothelial cells in the CNS or in cells that surround CNS capillaries, namely astrocytes. In contrast, iron accumulates in the brain in aceruloplasminemia in humans and mice (3–6), in which serum iron...
GPI-Cp is Required for Iron Efflux from the CNS

Figure 4. Iron transporters are expressed in astrocytes. mRNA expression of DMTI (A), IREG1 (B), and the ferric reductase Dcytb (C). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RT-PCRs were used as RNA controls. A, lane 1 shows DMT1 mRNA expression in rat astrocytes. Lane 2 shows wild-type CHO cells, and lane 3 shows CHO cells stably transfected with DMT1 cDNA (positive control). B, lane 1 shows IREG1 mRNA expression in rat astrocytes. Lane 2 shows rat duodenum (positive control) and lane 3 shows rat heart (negative control). C, lane 1 shows mRNA expression of the ferric reductase Dcytb in mouse astrocytes. Lane 2 shows mouse duodenum, and lane 3 shows heart (negative control). D, Western blots of rat astrocyte proteins showing DMT1 protein (65 kDa) in lane 1 and IREG1 protein (66 kDa) in lane 2. Equal protein loading was confirmed with anti-actin Western blots.

Figure 5. GPI-Cp is associated with IREG1. A, confocal microscopy of cultured astrocytes showing double-immunofluorescence labeling for cell surface GPI-Cp and IREG1. These two molecules are colocalized (arrows). B, In contrast, cell surface ceruloplasmin and DMT1 are not colocalized.

and serum transferrin saturation levels are very low (5, 6). We show here that a lack of ceruloplasmin expression by astrocytes leads to disruption of iron efflux. The requirement of GPI-Cp for iron efflux from astrocytes indicates that oxidation of ferrous iron transported across the cell membrane, probably via the transmembrane transporter IREG1, is an essential step. Recently, soluble ceruloplasmin was shown to be essential for iron transport across the oocyte membranes when GPI-Cp was expressed in these cells in vitro (16). Earlier studies on the yeast showed that a transmembrane multicopper oxidase Fet3 with homology to ceruloplasmin interacts with an iron permease, Ftr1p, to transport iron across the cell membrane (29, 30). We now show that IREG1 is expressed by astrocytes from the brain and is physically associated with GPI-Cp. Furthermore, we also provide evidence that the expression of IREG1 alone is insufficient to allow iron efflux in the absence of GPI-Cp. Because IREG1 transports ferrous iron that is highly toxic, ceruloplasmin as the major ferric oxidase in the CNS plays a crucial role in detoxifying it to the ferric state. The inability of IREG1 to efflux ferrous iron in the absence of ceruloplasmin may therefore serve as a protective mechanism to prevent efflux of toxic ferrous iron, leading to the rapid generation of free radicals. The slow accumulation of iron intracellularly may eventually surpass the ability of the intracellular sequestering capacity of the cell and lead to cell damage and death.

Because soluble ceruloplasmin in the CSF, which is produced by the choroid plexus, is extremely low (1 μg/ml compared with 300 μg/ml in the serum), it is likely to contribute minimally to the ferroxidase activity in the CNS. Astrocytes are known to be the only cell type in the CNS to express ceruloplasmin (20, 31), which is of the GPI-anchored form (8, 20). The severe accumulation of iron in the brain in cases of aceruloplasminemia indicates that ceruloplasmin expressed on the surface of astrocytes plays an important role in the maintenance of normal iron levels in the CNS and its mobilization out of the CNS. Furthermore, GPI-Cp expressed by astrocytes also seems to be capable of effluxing iron from neurons, because iron accumulates in neurons in aceruloplasminemia. How GPI-Cp regulates neuronal iron levels is not yet known but may involve the transfer of GPI-Cp from the astrocyte to the neuronal cell membrane, because GPI-anchored proteins can transfer from one cell to another by cell-to-cell contact (32). This might explain why Cp is found in both astrocytes and neurons in the CNS (33), whereas Cp mRNA is found only in the astrocytes (31, 33). One advantage of the GPI-anchored form or ceruloplasmin in the brain is that it reduces the need to have high levels of ceruloplasmin in the CSF. Our results indicate that at least 300 to 400 μg/ml of ceruloplasmin would be needed in the CSF to efflux iron from astrocytes. This contrasts sharply with the total protein concentration of 350 μg/ml in human CSF (34). Some of the other features that GPI anchors confer to proteins may also aid in the physiological function of ceruloplasmin; e.g., GPI-anchored proteins, which are located in lipid-rich microdomains, have a much greater degree of lateral mobility (35) and may also help serve as an apical targeting signal (36). The latter feature may lead to the targeting of GPI-Cp in vivo to the astrocytic end-feet that surround capillaries in the CNS and thus position the iron efflux mechanism near blood vessels via which iron can be mobilized out of the CNS.

This work provides the basis for understanding why iron accumulates in the CNS in aceruloplasminemia and may have...
implications for understanding the pathogenesis of other neurodegenerative diseases in which iron accumulation occurs, such as Parkinson’s disease, amyotrophic lateral sclerosis, and Alzheimer’s disease.

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